Final synopsis
For the Ph.D. thesis entitled

SCREENING OF PHYTOCHEMICAL CONSTITUENTS AND EVALUATION OF ANTICANCEROUS PROPERTIES OF *GYMNACRANTHERA FARQUHARIANA* (HOOK. F. & THOMSON) WARB.

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Chapter 1

1. Introduction

1.1 Family Myristicaceae

The plant family Myristicaceae is comparatively less explored and researched for its various pharmacological possibilities including the treatment for cancer. It consists of 19 genera with 400 species of trees and shrubs across the world (Mabberley, 2005). *Myristica* is the largest genus with 120 species and New Guinea has been reported to have a large number of species. The Genus *Virola* is dominant in Amazon Forest while *Knema* is distributed in South East Asia as reported by Takhtajan (1969) and Ahmedulla and Nayar (1986). In India, four genera viz., *Horsfieldia*, *Gymnacranthera*, *Knema,* and *Myristica* consisting of 15 species have been distributed along the evergreen forests of Andaman Nicobar Islands and Meghalaya.

The Western Ghats have three genera and five species; all of them are trees associated with evergreen to semi-evergreen forests. Of this, *Gymnacranthera farquhariana* and *Myristica fatua* var. *magnifica* are exclusive to the *Myristica* swamps, *M. malabarica* is occasional in the swamps and more frequently found in the evergreen forests. The other species are *M. dactyloides* and *Knema attenuata*. All the species except *Myristica dactyloides* are endemic to the Western Ghats (Ahmedulla and Nayar, 1986). The plants of the family are a source of various phytochemicals of prominence and utility concerning pharmacological considerations. The various members of the family like *Myristica andamanica, Knema attenuata, M. dactyloides, M. malabarica, M. fragrans* (Nutmeg), and *M. fatua* var. *magnifica* has been screened for their phytochemical constituents and pharmacological utility by various methods and interventions.

Gymnacranthera farquhariana is found in the swamp habitats of Uttara Kannada and Dakshina Kannada districts as well as in the *Myristica* wetlands of Gersoppa, Shimoga district, Karnataka. This plant co-dominates the vegetation of the *Myristica* marsh. Only marshy environments are connected with the species, and habitat degradation appears to be its main concern (Bhat, 2017). *G. farquhariana* has been not subjected to scientific screening and in-depth analysis of phytochemical vividity and utility for pharmacological possibilities especially concerning the anticancerous properties. So, the objective of this study deals with assessment of screening of phytoconstituents, antioxidant activity, *in vitro*, *in vivo* studies and LCMS analysis.

1.2 Cancer and phytochemicals:

Cancer is one of the most dreadful diseases that is globally distributed among the world's population. Cancer occurs due to excessive free radical damage, which ultimately causes damage to the genetic material DNA, protein, and lipids. This DNA damage leads to mutations that cause normal cells to transfer into a cancer cell (Roszkowski, 2014). Excessive production of free radicals occurs due to the increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). This leads to a condition of an increased oxygen environment called oxidative stress. In cancer cells, increased levels of reactive oxygen species have been found, which causes uninterrupted cell proliferation and leads to tumor development (Gorrini et al., 2013). Failure of apoptosis and increased rate of cell survival occurs due to DNA damage that results in cancer development. Apoptosis is the major form of programmed cell death, which takes place in all the cells to maintain homeostasis and cellular integrity. Cancer treatment therapies target this apoptotic pathway by increasing apoptosis in cells and thus preventing cancer (Kalimuthu and Se-Kwon, 2013; Zhao et al., 2014). The phytochemical studies of

most of the plants have also focused on screening and testing possible molecules to prevent and cure cancer by intervening positively at the molecular and genetic levels.

1.3 Rationale of the Present Work

Plant *Gymnacranthera farquhariana* is endemic and endangered species to Western Ghats. Habitat destruction has led to danger of extension of this plant (Ahmedulla and Nayar, 1986). The plant belongs to family Myristicaceae which has 19 genera with 400 species of trees and shrubs across the world (Mabberley, 2005). Most of the plants of the family have been detailed with respect to its phytochemical, pharmacological uses. (Patro et al., 2005; Thuong et al., 2013; Ginting, 2021). Folklore or pharmacological utility of the plant *G. farquhariana* has not been established (Chaithanneya and Bhat, 2016; Bhat 2017).

Phytochemical screening utilizing different parts of the plant *G. farquhariana* has not been subjected to satisfy a comprehensive screening. The plant is screened for phytochemical properties in only methanol and aqueous extracts an exhaustive screening using multiple solvents is desirable. Pharmacological utility of *G. farquhariana* has been identified for its antidiabetic, antimicrobial, anti-inflammatory and antioxidant properties (Chaithanneya and Bhat, 2016; Bhat, 2017; Anusha et al., 2019).

The phytochemical screening is limited to identification of different groups of compounds, in-depth analysis of these compounds through LCMS has been not taken up. *In vitro* studies have been taken up to establish anticancer activity in limited cell lines (Bhat, 2017). *In vivo* studies have been suggested to establish antitumor property of the plant but has been not taken up yet (Chaithanneya and Bhat, 2016; Bhat, 2017; Anusha et al., 2019).

Cancer is the deadliest medical problem and a research challenge. The curing pathways evolved are limited by side effects and suffering of the patients since most of it is based on synthetic molecules. Plant based ethnomedical practices have been impressive in cancer cure, plant derivatives have been strongly recommended for synthesis of anticancer molecules to replace synthetic molecules (Birjees et al., 2021; Garcia-Oliveira, 2021; Chaachouay et al., 2022).

Most plants of the family Myristicaceae have been screened and active molecules have been identified with respect to pharmacological utility but for *G. farquhariana.* The presence of active molecules has been strongly associated with anticancer and antitumor property among other plants of the family. *G. farquhariana* to be screened for possibility of presence of similar compounds with better potency of anticancer property in *in vivo* and *in vitro* is models.

Identification and isolation of active molecules in association with antitumor property has been not the part of any studies till now. Association between molecule *in vivo* activity and cancer curing pathway necrosis / apoptosis has also not been established. Phytochemical screening identification of active molecules *in vivo* and *in vitro* evaluation of them with respect to anticancer and antitumor activity ensuring cytotoxic limitations and molecule specific cancer healing pathways is desirable with respect to the extinct rare plant *G. farquhariana.*

Graphical abstract of the current study is shown in figure 1. The seeds contain fat which were used for making candles in earlier days but other uses have not been recorded (Anonymous, 1962). There is no

information regarding the usage of different parts of *G. farquhariana* in Traditional Knowledge Digital Library (TKDL).

Objectives

1. To analyse and record the phytochemical constituents of leaf, bark, and seeds of *Gymnacranthera farquhariana* using different solvent extracts.
2. To study the antioxidant activity and to evaluate the effect of the extracts on the cancer cell lines (*in vitro*).
3. To evaluate the crude extract of *G. farquhariana* for antitumor properties using balb/c mice.
4. To study the histomorphology changes of induced tumor in balb/c mice.

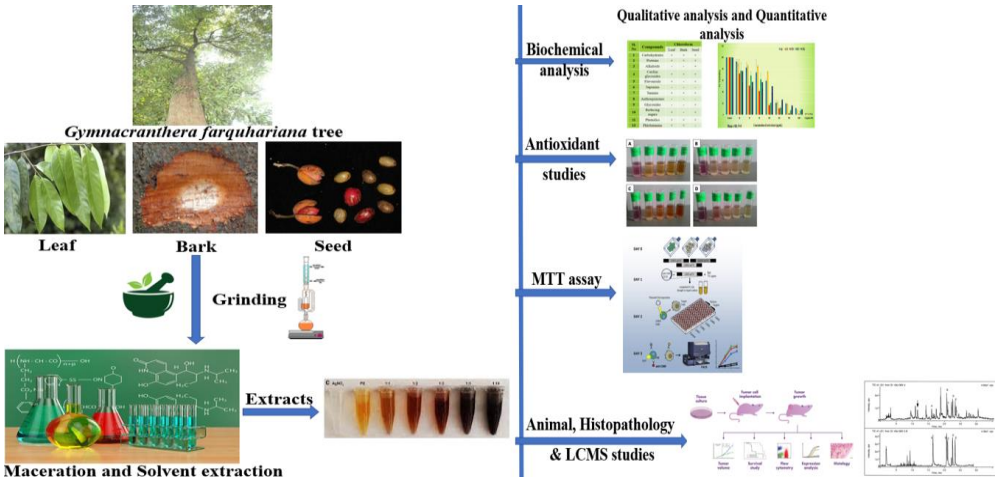


Figure 1: Graphical abstract

Chapter 2

2. Review of Literature

2.1 Phytochemical studies of the family Myristicaceae:

The phenolic compounds extracted from the seed resin of *M. malabarica* were reported to be good antioxidants among various edible oils and fats compared to butylated hydroxytoluene as published by Duggal and Kartha (1956). New lignin, attenuol (58%) was isolated from the hexane extract of bark of *K. attenuata* by Joshi et al. (1977). The chromatography of the nutmeg of *M. fragrans* by Gopalakrishnan and Mathew (1983) reported the presence of epicatechin and cyanidin. The presence of saponins, polyphenols, tannins, epicatechin, triterpenic sapogenins, and fats was reported in the seeds of *M. fragrans* by Varshney and Sharma (1968) and Sathyavathy et al. (1987). The presence of alkaloids, glycosides, and steroids was also confirmed by thin-layer chromatography. The dichloromethane and ethanolic extracts of *K. angustifolia* stem showed the presence of condensed tannins, phenolic compounds, and triterpenes reported by Phadungkit et al. (2010). Koperuncholan and John (2011) reported the presence of flavonoids, phenolic compounds, saponins, tannins, triterpenoids, steroids, and importantly the absence of alkaloids, anthraquinones, and amino acids in different solvent extracts in *M. dactyloides*.

The presence of phenolic contents in different extracts of seed and aril of *K. attenuata* fruits and leaves and fruits of *Myristica fragrans* was reported by Vinaychandra and Chandrashakar (2014) and Shazia et al. (2015). There are works of literature on the presence of a wide variety of phytoconstituents in the

members of Myristicaceae plant parts using various solvent systems as reported by (Chakraborty et al., 2015; Chaithanneya and Bhat, 2016; Tantry and Bhat, 2016; Vivek and Chandrashekar, 2016; Prabha et al., 2018; Anusha et al., 2019).

2.2 Antioxidant and cell line studies of members of family Myristicaceae:

The phenolic compounds present in the seed resin of *M. malabarica* were found to be good antioxidants in various edible oils and fats than butylated hydroxy toluene as reported by Duggal and Kartha (1956).

According to Zheng et al. (1992), Myristicin is found in the volatile oil of *M. fragrans* acts as a cancer chemo preventive agent. According to Kumari (1992), *M. fragrans* mace protected from bone marrow genotoxicity in male Swiss albino mice. The ethanol extract showed a strong cytotoxicity activity against the lung cancer cell line with an IC_{50} value of 4.55 ± 4.60 µg/mL. Dorman et al. (2000) reported that nutmeg essential oils are powerful antioxidants and showed activity of 5.00 ± 2.14 m mol/L at 1000 ppm. Gokhale et al. (2004) determined the chemical constituents of *M. malabarica* and reported the presence of myristic, palmitic, and oleic acids. Patro et al. (2005) isolated a new 2-hexylresorcinol and four known diarylnonanoids (Malabaricane A-D) from *M. malabarica* of which diaryllnonanoid, malabaricone C has been reported to be shown the maximum DPPH scavenging activity. Also, they found that malabaricone C could prevent both Fe (II) and 2, 2 azobis (2-amidinopropane) dihydrochloride induced lipid peroxidation of rat liver mitochondria more efficiently compared to curcumin.

Aril of *M. fragrans* has been reported to be shown significant antioxidant activity due to its ability to inhibit lipid peroxidation and superoxide radical scavenging activity in the rat as reported by Yadav and Bhatnagar (2007). In the anticancerous study conducted by Chirathaworn et al. (2007) at the concentration 50 and 100 µg/mL, the methanol extract of *M. fragrans* significantly inhibited Jurkat cell proliferation and induced apoptosis as detected by annexin 5 staining. Downregulation of SIRT1 mRNA expression in Jurkat cells was observed even when the amount of methanol extract was 10 µg/mL.

Duan et al. (2009) studied the antioxidative activities against 2,2-diphenyl-1-picrylhydrazyl radical and cytotoxicity against K-562 cells were tested, and (7S,8S,7´R,8´S)-4,5´-dihydroxy-3,3´dimethoxy-7,7´-epoxylignan showed the corresponding activities with IC_{50} values of 39.4 and 2.11 µM respectively. Later in the seed oil, antioxidant activities in six compounds, viz, licarin-B, dehydrodi isoeugenol, malabaricone B, malabaricone C, β-sitosterol, and daucosterol were found and reported that malabaricone B was identified for the first time in nutmeg. It was proved that malabaricone C is an efficient antioxidant agent since it exhibited stronger antioxidant activity than the commonly used synthetic antioxidants will lead to preventing cancer by the dietary intervention (Hou et al., 2012).

Piras et al. (2012) evaluated both the essential oils obtained from nutmeg for cytotoxicity (MTT assay) in a human colon adenocarcinoma cell line (undifferentiated Caco-2 cells), extensively used in the studies of anticancer activity of natural active extracts and compounds. Both the essential oils showed a similar pattern, exerting a significant reduction superior to 60% in cell viability from the concentration of 250 µg /mL. The IC_{50} values were 167 and 155 µg/mL for HD and SFE 90 respectively. The cytotoxicity of the main essential oil component, myristicin was also tested for its activity. Helen et al. (2012) analysed the cytotoxic effects of oil extract on MCF-7 and A375 cell lines by trypan blue dye exclusion method. Methanolic extract of the stem, bark of *M. malabarica* showed a significant dose-

dependent antioxidant activity with IC_{50} values at 0.02 mg/mL in DPPH assay (Manjunatha et al., 2012).

Thuong et al. (2013) isolated four lignans named meso-dihydroguaiaretic acid (DHGA), macelignan, fragransin A2, and nectandrin B, from the seeds of *M. fragrans* (Vietnamese nutmeg) and investigated for their cytotoxic activity against eight cancer cell lines like H1299 (human non-small cell lung carcinoma), H358 (human bronchiolar lung cancer), H460 (large cell lung cancer), Hela (human cervical cancer), HepG2, KPL4, MCF-7, RD, and MDCK (Multicystic dysplastic kidney). Among these DHGA exhibited potent cytotoxicity against H358 with an IC_{50} value of 10.1μM. In addition to this, it showed antitumor activity in the allogeneic tumor-bearing mice model, further confirming the anticancerous potential by animal experiment.

Gupta et al. (2013) investigated the antioxidant and antimicrobial activities of nutmeg (*Myristica fragrans* Houtt.) the seeds were extracted with acetone, ethanol, methanol, butanol, and water. All the extracts have shown significant antioxidant and antimicrobial activities against the tested microorganisms. Among all extracts, acetone extract had shown the highest antioxidant activity. Sabinene (28.61%) has shown the highest occurrence in the extract. b-Pinene (10.26), a-pinene (9.72), myristicin (4.30%), isoeugenol (2.72%), p-cymene (1.81%), carvacrol (1.54%), eugenol (0.89%), and b-caryophellene (0.82%) was reported as a possible contributor for antioxidant and antimicrobial activity of nutmeg, proving the possibilities of further studies and utilizing it for the medicinal purpose. Petroleum ether extract of *M. dactyloides* bark showed a potent DPPH scavenging activity at the concentration of 10 μg/mL as reported by Ajjsh et al. (2015).

Shazia et al. (2015) conducted a phytochemical analysis of *Myristica fragrans* leaves by using different solvents like aqueous, cyclohexane, benzene, chloroform, acetone, ethyl acetate, ethanol, and methanol and reported the study revealed the antioxidant activity of different extracts was, and the highest activity was reported in methanol extract and obtained IC_{50} value of 56.83±5.67 μg/mL by DPPH method. Similar observations were made by Chowdhury et al. (2017).

A significant IC_{50} value was reported in the bark, kernel, aril, and fruit rind extracts of *M. fatua* var. magnifica, methanolic, and ethyl acetate extracts of the kernel, methanolic extract of aril and ethyl acetate of testa also shown significantly higher on par DPPH activity (Vivek and Chandrashekar, 2016). However, the leaf extracts did not show any significant DPPH scavenging activity. Tantry and Bhat (2016) evaluated the methanolic and aqueous seed extracts of *Myristica fatua* var. *magnifica* whereas the methanolic extract showed positive results for carbohydrates, proteins, alkaloids, phenolic compounds, tannins, and resins, and a greater quantity of compounds were found in methanol as compared to the aqueous extract. The methanolic extract showed significant antioxidant activity.

A study undertaken by Chaitanneya and Bhat (2016) proved that the methanol and aqueous extracts of seed of *Gymnacranthera farquhariana* have the presence of carbohydrates, proteins, phenolics, resins, tannins, and alkaloids. The same study showed that methanol extract had precise antimicrobial activity compared to aqueous extract. The methanolic extract also had exhibited antioxidants. Bhat, (2017) also proved the cytotoxicity activity using leaf and seed extract with cell line HL-60. Anusha et al. (2019) evaluating the *G. canarica* seeds extract at the dose of 100 μg/mL shows 22.3% the significant free radical scavenging activity of DPPH. A noticeable concentration-dependent increase in the inhibition percentage cannot be seen.

2.3 Animal studies for potent anticancerous properties of plant extracts

Jannu et al. (1991) by using the cervical carcinogenesis model system, *M. fragrans* mace was administered orally at the dosage of 10 mg/kg for 7 days later they found as compared with that of the control (73.9%), was 21.4% reduction. This decline in the incidence of carcinoma was highly significant ($P < 0.001$).

Othman et al. (2011) investigated the therapeutic effect of ethanolic neem leaf extract in an *in vivo* 4T1 breast cancer model in mice. Cancerous mice treated with 0.5 μg/mL tamoxifen citrate (CT), 250 mg/kg neem leaf extract (CN 250) or 500 mg/kg neem leaf extract (CN 500). By TUNEL assays it was proved that CN 250 and CN 500 groups had a higher incidence of apoptosis compared with the cancer controls.

Lal et al. (2012) investigated the effects of piperine, a major pungent alkaloid presents in *Piper nigrum* and *P. longum*, on the tumor growth and metastasis of mouse 4T1 mammary carcinoma *in vitro* and *in vivo*, and elucidated that Piperine (35–280 μmol/L) inhibited the growth of 4T1 cells in a time- and dose-dependent manners, at 48 and 72 h. The cells treated with piperine (140 and 280 μmol/L) significantly increased the percentage of cells in the G2/M phase with a reduction in the expression of cyclin B1. Piperine (140 and 280 μmol/L) significantly decreased the expression of MMP-9 and MMP-13 and inhibited 4T1 cell migration *in vitro*.

Thuong et al. (2013) *M. fragrans* lignans, meso-dihydroguaiaretic (2mg/kg, 5mg/kg and 10mg/kg) (DHGA) was administrated to tumor- bearing mice at 10mg/kg, the effective reduction of tumor was noticed

Luo et al. (2014) had investigated the antitumor, anti-metastasis, and anti-osteolytic effects of Green tea (*Camellia sinensis*, CS), CS aqueous extract both *in vitro* and *in vivo* using metastasis-specific mouse mammary carcinoma on 4T1 cells. They reported that treatment of 4T1 cells with CS aqueous extract resulted in significant inhibition of 4T1 cell proliferation. CS extract induced 4T1 apoptosis in a dose-dependent manner as assessed by annexin-V and propidium iodide staining and caspase-3 activity.

Yazan et al. (2015) determined the anti-breast cancer activities and the safety oral consumption of *Dillenia suffruticosa* root aqueous extract (DRAE) in BALB/c mice, proved that DRAE possesses anti-breast cancer properties but at the same time it shows mild toxicity to the liver.

In a study conducted by Narayanan et al. (2015) on Melinjo (*Gnetum gnemon* L.) seed extract (MSE) and its active ingredient gnetin C (GC), a resveratrol dimer, have been shown to possess a broad spectrum of pharmacological activities. In this study, they investigated the antitumor activity of MSE and GC using human and murine tumor cell culture models *in vitro*. The antitumor activity of GC was compared with trans-resveratrol (TRV), a stilbenoid polyphenol. Their results showed that MSE and GC at clinically achievable concentrations significantly inhibited the proliferation of pancreatic, prostate, breast, and colon cancer cell types ($P< 0.05$), without affecting.

Bagheri et al. (2017) demonstrated that asafoetida has potent antitumor and antimetastasis effects on breast cancer and is a potential source of the natural antitumor product. The antitumor effect of asafoetida was studied using *in vivo* mouse mammary carcinoma 4T1 cells on female BALB/c mice

apart from the antitumor effect, asafoetida decreased lung, liver, and kidney metastasis and increased areas of necrosis in the tumor tissue respectively.

In the investigation conducted by Alipanah and Zareian, (2018) the inhibitory effect of *Boswellia serrata* gum resin alcoholic extract (BSE) on tumor growth, metastasis, and angiogenesis in 4T1 breast cancer mouse model in which the female BALB/c mice was treated by BSE (50,150 and 250 mg/kg) and distilled water for 21 days. Antiproliferation and anti-angiogenesis effects of BSE in tumor tissues were evaluated by immune histochemical (IHC) analysis for Ki-67 and CD31 expression. In an *in-vitro* toxicity study, 4T1 cells line was reported to be sensitive to BSE treatment with reduced cell viability. BSE suppression of 4T1 tumor growth correlated with reduced cell proliferation as revealed by IHC analysis for Ki-67 expression.

Aumeeruddy and Mahomoodally, (2019) studied the three plants which have received great attention concerning their anticancer properties: 1) piperine, a dietary phytochemical isolated from black pepper (*Piper nigrum* L.) and long pepper (*Piper longum* L.) 2) sulforaphane, an isothiocyanate mainly derived from cruciferous vegetables; and 3) Thymoquinone, the active compound from black seed (*Nigella sativa* L.). The combined effect of these 3 compounds on conventional cancer therapy to observe enhanced efficacy compared with single treatments was studied and proved significant.

Chapter 3

3. MATERIALS AND METHODS

3.1 Collection and authentication of plant materials

The leaf, bark, and seeds were collected from Panja of Sullia taluk of D.K District, Karnataka, India. The plant was identified and authenticated by Dr. Rama Bhat, Professor of the PG Biotechnology Department at Alva's College, Moodbidri affiliated to Mangalore University, Karnataka. The herbarium was verified at Botanical Survey of India, Coimbatore, Tamil Nadu. The herbarium was deposited in PG department of Alva's with the herbarium number ALCM/BT/10. Seeds were separated from the fruits gathered during the fruiting season i.e., August. The latitude and longitude of the place are 13˚.01' 45.9" N, 74˚.49'24.3" E. Leaf, bark, and seeds were dried in shade, and shade-dried samples were milled to obtain a coarse powder. The initial and final weight of this coarse powder was calculated.

3.2 Preparation of solvent extract

About 15g of dried leaf, bark, and seeds were crudely powdered and subjected for extraction in soxhlet extractor in different polar solvents like aqueous, acetone, ethanol, methanol, and chloroform. All the extracts were concentrated, and the left-over solvent was evaporated to dryness using a water bath (Mohanta et al., 2007; Hoshyar et al., 2015).

3.3 Phytochemical screening:

3.3.1 Qualitative tests: Qualitative tests like carbohydrates, proteins, alkaloids, cardiac glycosides, flavonoids, saponins, tannins, anthraquinones, glycosides, reducing sugars, phenolics, and phlobatanins were carried out on the aqueous, acetone, ethanol, methanol, and chloroform extracts with the standard protocols (Sadasivam and Manickam,2008).

3.3.2 Quantitative test: Quantitative test is carried out on the aqueous and methanol extracts of leaf, bark, and seed with the standard protocols (Sadasivam and Manickam, 2008). All the phytoconstituents were expressed as mg/g of dry weight of plant material.

3.3.3 Estimation of proteins: The total protein estimation was conducted according to the standard method described by Lowry et al. (1951).

3.3.4 Estimation of phenolic content: The total phenolic content present in each of the aqueous and methanolic extracts of leaf, bark, and the seed was evaluated by the Folin-Ciocalteau method (Singleton et al.,1999).

3.3.5 Estimation of reducing sugars by Dinitro salicylic acid (DNS) method: The concentration of the reducing sugar present in the leaf, bark, and seed was estimated by Dinitro salicylic acid method (Sadasivam and Manickam, 2008).

3.3.6 Estimation of tannin: The tannin content was determined by Folin-Denis's method (Sadasivam and Manickam, 2008).

3.3.7 Antioxidant potential of aqueous and methanol extracts of leaf, bark, and the seed of *G. farquhariana* by using DPPH radical scavenging method (Zuraini et al., 2008; Nithianantham et al., 2011).

3.4 MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

The human normal kidney cell line (HEK 293), human breast cancer cell line (MCF-7) human lung cancer cell line (A549), and mouse breast cancer cell line (4T1) were used for the study. The cells were routinely maintained in Minimum Essential Medium (MEM), Ham's F12 Medium, and Roswell Park Memorial Institute (RPMI) media in a humidified chamber (95%) with 5% CO_2. The cultures were routinely maintained by trypsinization of existing cells and transferring them to the new flask or using it for experimental purposes. Subculture was performed once the cells in the flask reached 90% confluence. Cytotoxicity was analysed using MTT assay following the protocol described by Mosmann, (1983). Cisplatin used as standard drug. The quantity of formazan was measured through the plate reading in spectrophotometer-based absorbance at 570 nm.

The percentage of inhibition of growth was calculated using the formula

$$\% \text{ Viable cells} = [(\text{Test sample} - \text{Blank}) / (\text{Control} - \text{Blank})] \times 100$$

3.5 Animal studies

Healthy female BALB/c mice of 6-8 weeks, weighing about 25 g, were obtained from Manipal School of Life Sciences, MAHE was used in the present study. Animals were collected from the breeding colony and acclimatized to the laboratory condition for 2 weeks. The animals were maintained in a controlled environment condition of temperature (23 ± 2^{o}C) and humidity (45-50%) on alternatively 12 hours light/dark cycles. They were kept in standard polypropylene rat cages with stainless steel top grill supplied by Inco, India, with sterilized paddy husk as the bedding material. The animals were fed with a standard commercial diet and had free access to water during the experiments. Experiments were performed complied with the rulings of the Committee for Control and Supervision of Experiments on Animals by Institutional Animal Ethics Committee *(*IAEC*),* Kasturba Medical College, MAHE, Manipal (Reg. No. IAEC/KMC/80/2019).

A total of 24 female BALB/c mice were divided at random into 4 groups (6 animals per group). Each mouse's lower flank region was shaved properly, to the subcutaneous region 50 lakhs cells, 4T1 mice breast cancer cells were injected to anesthetized animals. After 20 days of breast cancer cell

implantation, animals were orally administered 25mg/kg with plant extract and standard drugs respectively for 15 days. Group 1 tumor control, Group 2 Methanol seed extract, Group 3 Chloroform seed extract, and Group 4 Cyclophosphamide standard drug. Tumor measurements like length, breadth, and height were noted on alternative days (Hussain and Rao, 1991; Thuong et al., 2013; Yazan et al., 2015). After the administration of the last dose, each group was sacrificed for histopathological studies. Tumor tissues were processed and stained using the hematoxylin and eosin staining techniques **(**Suvarna et al., 2019).

3.6 LC-MS analysis: An untargeted LC-MS analysis was performed to identify different analytes present in the extract using the Q-TOF MS instrument.

Chapter 4

4. RESULTS AND DISCUSSION

4.1 Qualitative tests

Cancer is a disease that is characterized by unchecked cell development and, on occasion, the spread of these aberrant cells to other bodily regions. In majority of the countries, it is the main cause of death mainly observed that in nations with low and medium incomes (LMICs) there is a rise in non-communicable diseases like cancer compared to communicable diseases Kanavos (2006).

Singh et al. (2016) reported that alkaloids, flavonoids, phenolics, tannins and glycosides are lead phytochemicals used for anticancer drug development. In the present study, all these phytochemicals were detected in the leaf, bark and seed extracts of *Gymnacranthera farquhariana*.

In the current investigation, bioactive chemicals were extracted using chloroform, ethanol, acetone, methanol, and water, taking into account all of the aforementioned factors. Among solvents, methanol extracted maximum number of phytochemicals compared to other solvents. Norsyamimi et al. (2014) reported that methanol is a commonly used extractant because of its strong polarity and ability to create large amounts of bioactive chemicals. Truong et al. (2019) conducted a study on influence of solvents on bioactive molecule production from *Severinia buxifolia.* Their research unequivocally demonstrates that methanol was the best solvent for removing bioactive chemicals from plants since it produced a high yield of phenolics, flavonoids, terpenoids, and alkaloids. They also found that the anti-inflammatory and antioxidant effects of methanol extract were very high in methanolic extract compared to other extracts. According to Do et al. (2014), compared to other solvents, 100% ethanol extracted the most total phenolic and flavonoid content from *Limnophila aromatica*. Water as a solvent is safe, ecofriendly, easily accessible, cheap compared to other organic solvents (Vong et al., 2014). Methanol is regarded by the U.S. Food and Drug Administration (FDA) as a secure solvent for the extraction of bioactive substances (Anonymous, 2018). Based on these findings and recommendations, methanol and water were utilized in the current study as solvents to extract the bioactive components from *Gymnacranthera farquhariana*.

Table 4.1 reveals that alkaloids are extracted in ethanol and methanol solvents better when compared to other solvents. Cardiac glycosides are found more in chloroform and methanol extracts. Flavonoids are better extracted in methanol. In the case of saponins, it is ethanol and aqueous extracts found to be effective. Tannins are found in chloroform and aqueous solvents in abundance compared to other solvents. Anthraquinones are extracted better in acetone and methanol extracts. Glycosides are extracted through ethanol in more samples. Glycosides are also better extracted in ethanol. For reducing

sugars, it is methanol as a solvent that has better extraction. Phenolics are found more in methanol and chloroform solvents and phlobatanins are found more in chloroform.

Arunachalam and Subhashini (2010) reported the presence of amino acids, carbohydrates, alkaloids steroids, glycosides, and lipids in *Myristica andamanica*. In the methanolic leaf and seed extract of *Gymnacranthera farquhariana*, Chaithanneya and Bhat (2016) observed the presence of proteins, alkaloids, tannins, phenols, carbohydrates, and resins. Similarly, in the aqueous and methanolic seed extract of *Myristica fragrans*, Shazia et al. (2015) found chemical constituents like alkaloids, flavonoids, tannins, glycosides, reducing sugars, steroids and terpenoids. The present study also revealed the presence of similar phytochemicals as compared to the earlier studies. The *M. fragrans* leaf methanol and acetone solvent (1:7) has demonstrated the existence of flavonoids, alkaloids, terpenoids, steroids, saponins, glycosides, and tannins (Muthanna et al., 2022).

Table 4.1: Phytoconstituents in leaf, bark, and seed extract of *G. farquhariana* in different solvents

Sl. No	Test	Chloroform			Ethanol			Acetone			Methanol			Aqueous		
		Leaf	Bark	Seed	Leaf	Bark	Seed	Leaf	Bark	Seed	Leaf	Bark	Seed	Leaf	Bark	Seed
1	Carbohydrates	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	Proteins	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	Alkaloids	-	-	+	+	+	-	-	-	+	-	+	+	+	-	-
4	Cardiac glycosides	+	+	+	-	+	+	+	-	+	-	+	+	-	-	-
5	Flavonoids	+	-	-	+	-	+	+	-	+	+	-	+	+	-	+
6	Saponins	-	-	-	+	+	-	-	+	-	+	-	-	+	+	-
7	Tannins	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+
8	Anthraquinones	-	-	-	-	+	-	-	+	+	+	+	-	-	+	-
9	Glycosides	-	-	+	-	+	+	-	-	+	-	-	+	-	-	-
10	Reducing sugars	+	-	+	-	+	-	-	-	+	+	+	-	+	-	-
11	Phenolics	-	+	-	+	-	+	-	-	+	-	+	+	+	+	+
12	Phlobatanins	+	+	-	+	-	-	-	+	-	-	-	-	-	-	-

Presence + Absence -

4.2 Quantitative test

The protein content is maximum in the bark and seed extract using aqueous solvent (8.53±0.85 mg/g) and (5.55±0.82 mg/g) respectively. Phenolic content is found to be maximum in seed extract of methanol solvent (1.53±0.024 mg/g). Reducing sugars was found to be maximum in leaf extract in methanol (5.34±0.391mg/g). Tannic acid was also found to be maximum in the seed extract of methanol (2.06± 0.016 mg/g). Maximum amount of phenolics in seed (1.53±0.02 mg/g), reducing sugars in leaf (5.35±0.39 mg/g) and tannins in seed (2.07±0.01 mg/g) was reported in methanol solvent and protein (8.52± 0.85 mg/g) is extracted in aqueous bark (Table 4.2).

Table 4.2: Total protein, phenolics, reducing sugar and tannic acid in leaf, bark, and seeds of *G. farquhariana*

Plant parts	Protein (mg/g)		Phenolic (mg/g)		Reducing sugar (mg/g)		Tannic acid (mg/g)	
	Aqueous	Methanol	Aqueous	Methanol	Aqueous	Methanol	Aqueous	Methanol
Leaf	3.15± 0.3	6.25± 0.84	0.49 ± 0.012	0.755 ± 0.024	3.5 ± 0.273	5.346 ± 0.391	0.51± 0.012	1.82± 0.053
Bark	8.524± 0.85	7.574 ± 0.17	0.605± 0.01	1.38 ± 0.037	3.274 ± 0.356	3.598 ± 0.328	1.03± 0.068	1.66± 0.037
Seed	5.548 ± 0.82	7.372 ± 0.35	0.565 ± 0.054	1.53 ± 0.024	3.474±0.278	4.72 ± 0.193	0.53 ± 0.018	2.075± 0.016

Mean ± SD; N=5

Table 4.3 represents protein concentrations (mg/g) in different parts extracted using different solvents. The mean protein contents differed significantly at a 1% level between different parts of plants. The proteins extracted by the solvent systems also differed significantly at the 1% level. Amongst treatment combinations, proteins extracted by water from the bark of the plant (0.854mg/g) are significantly higher than other treatment combinations. The protein concentration recorded in the water leaf combination recorded significantly lowest protein concentration than other treatment combinations (0.314mg/g).

Lavee and Avidan (1993) reported that bark stores more water-soluble protein than a leaf with respect to olive plants. It has been observed that protein storage vacuoles are observed in bark and absent in leaves. These vacuoles are meant for the storage of proteins (Wetzel et al., 1991). This literature suggests that more water-soluble proteins are observed in bark than in leaves. According to Hew et al. (2013), MDA-MB-231 breast cancer cell line proliferation was inhibited by water-soluble proteins isolated from *Gynura procumbens* (Lour.) Merr. with an EC50 value of 3.8 g/mL. They also noticed that MDA-MB-23 cells treated with water-soluble protein fractions SN-F11/12 greatly decreased the mRNA expression of the proliferation markers Ki67 and PCNA.

Table 4.3: Concentrations of protein (mg/g) in different parts of *G. farquhariana*

Solvents (Factor A)	Protein concentrations in plant parts (Factor B) (mg/g)			
	Leaf	Bark	Seed	Mean
Aqueous	0.31	0.85	0.55	0.57
Methanol	0.62	0.75	0.73	0.70
Mean	0.46	0.80	0.64	

Factors	SEm±	CD (0.01)
A	0.02	0.01
B	0.03	0.02
AXB	0.04	0.03

Table 4.4 represents the Concentration of phenols (mg/g) in different parts of *Gymnacranthera farquhariana*. In general, methanol extract exhibited significantly more amount of phenols (mean phenol content of 0.141mg/g) than water extract (mean phenol content of 0.317mg/g). Among plant parts, extracts of seed showed significantly higher concentrations of phenols (0.270 mg/g) than extracts

of the other two plant parts (0.161mg/g and 0.255mg/g respectively in leaf and bark extracts respectively).

Among plant part and solvent combinations, the methanol extract of seeds showed significantly the highest amount of phenol (0.396 mg/g) than other combinations whereas, the aqueous extract of leaf showed a significantly lowest concentration of phenol (0.126mg/g). Because a compound's structure affects the polarity level, conjugation, and interaction of the sample matrix, it has been found that the structural variations of phenolic compounds have an impact on their solubility and separation capabilities (Alara et al., 2021). Aqueous methanol was also found to be the most efficient extractant for polyphenols. According to Do et al. (2014), methanol is the most efficient extractant in extracting phenols and polyphenols (Iloki-Assanga et al., 2015).

Table 4.4: Concentration of phenols (mg/g) in different parts of *G. farquhariana*

Solvents (Factor A)	The concentration of phenols in plant parts (Factor B) (mg/g)			
	Leaf	Bark	Seed	Mean
Aqueous	0.12	0.15	0.14	0.14
Methanol	0.19	0.35	0.39	0.31
Mean	0.16	0.25	0.27	

Factors	SEm±	CD (0.01)
A	0.002	0.007
B	0.003	0.008
AXB	0.004	0.012

The concentration of reducing sugars (mg/g) in different parts of *Gymnacranthera farquhariana* are represented in table 4.5. The concentrations of reducing sugars differed significantly concerning solvents and plant parts used. Methanol extracted significantly the highest amount of reducing sugars (0.235mg/g) compared to water (0.174mg/g). Among the plant parts, the leaf exhibited a significantly high amount of reducing sugars (0.227mg/g) compared to seeds (0.210mg/g) and bark (0.176 mg/g). The leaf samples with methanol extractant exhibited maximum reducing sugars (0.227mg/g) compared to any other treatments.

Table 4.5: Concentration of reducing sugars (mg/g) in different parts of *G. farquhariana*

Solvents (Factor A)	The concentration of reducing sugars in plant parts (Factor B) (mg/g)			
	Leaf	Bark	Seed	Mean
Aqueous	0.17	0.16	0.17	0.17
Methanol	0.27	0.18	0.24	0.23
Mean	0.22	0.17	0.21	

Factors	SEm±	CD (0.01)
A	0.005	0.01
B	0.006	0.01

AXB	0.008	0.02

The concentration of tannins (mg/g) in different parts of *Gymnacranthera farquhariana* is represented in table 4.6. The concentrations of tannins differed significantly for solvents and plant parts used. Methanol extracted significantly the highest amount of tannins (1.011mg/g) compared to water (0.337 mg/g). Among the plant parts, seeds exhibited a significantly high amount of tannins (0.781mg/g) compared to bark (0.660mg/g) and leaf (0.581mg/g). The seeds sample with methanol as an extractant exhibited maximum tannins (1.3 mg/g) compared to any other treatments. According to Naima et al. (2015), methanol is superior to methanol and water as a solvent for extracting tannins.

Table 4.6: Concentration of tannins (mg/g) in different parts of *G. farquhariana*

Solvents (Factor A)	**The concentration of reducing sugars in plant parts (Factor B) (mg/g)**			
	Leaf	**Bark**	**Seed**	**Mean**
Aqueous	0.25	0.49	0.26	0.33
Methanol	0.90	0.82	1.30	1.01
Mean	0.58	0.66	0.78	

Factors	**SEm±**	**CD (0.01)**
A	0.01	0.03
B	0.01	0.03
AXB	0.01	0.05

Higher protein content of 8.524± 0.85 mg/g was observed in bark aqueous extract. A previous study (Bhat and Kaveriappa, 1998) on the proximate analysis of the kernel and mace of *M. fatua* var. *magnifica* found that the total sugar content was 9.8% for the kernel and 19.3% for the mace, as well as 14.1 mg/mL of protein for the kernel and 11.6 mg/mL for the mace, and 8.9% for tannin. The tannin content of *M. fragrans* seeds in methanol and n-hexane extract was 486.57 ± 0.06 and 427.61 ± 0.06 (mg/100g) respectively (Anaduaka et al., 2022).

In the current investigation, the reducing sugar in methanolic leaf extract is 5.346 ± 0.391 mg/g and tannin content are 2.075± 0.016 mg/g in methanolic seed extract. On the other hand, nutmeg (*M. fragrans*) seed analysis using acetone extract, reported phenolic equal to 0.6217 mg/mL by Muthanna et al. (2022). Raja and Suku (2019) found total phenolic content of 1000 µg ml^{-1} (387.6 µg ml^{-1}gallic acid equivalents) ethanolic bark extracts of *K. attenuata* and 563.45 ± 0.06 *(*n-hexane extract) and 626.56 ± 5.77 (methanol extract) (mg/100g) in *M.* fragrans seeds (Anaduaka et al., 2022). Anusha et al. (2019) evaluated the total phenolic content in the mature seeds of *G. canarica* and reported 8.75 µg GAE/mg in methanolic extract. Vivek and Chandrashaker (2016) reported total phenolics in *M. fatua* which ranged between 14.67 and 195 mg GAE/mg. According to Chowdhury et al. (2017), the methanolic seed extract of *M. fragrans* contained 186.25 mg/g of total phenols, which is equal to gallic acid. In the current investigation, the highest total phenolic content of the seed methanol extract was 1.53 0.024 mg/g

4.3 Antioxidant studies

One of the reasons for development of cancer is presence of abnormally high concentrations of reactive oxygen species (Valko et al., 2007) which are naturally formed in the body and are responsible for

oxidative damage of major components of cells, DNA and proteins. Under normal conditions, they play an important role in many normal cellular processes. Antioxidants - a special category of substances delay or inhibit the oxidation by reactive oxygen species and thereby preventing cellular damages and disease preclusion (Rakesh et al., 2010; Kapadia et al., 2016). Our Body synthesize internal anti-oxidants and prevent damage caused by reactive oxygen species. On the other hand, there are some antioxidants called exogenous antioxidants are those that come from outside of biological systems.

Recently, there is an increasing awareness and widespread research on exogenous antioxidants from natural sources since they are more affordable, easily accessible, and thought to have fewer or no negative effects than their synthetic equivalents (Tadhani et al., 2007). Considering these points, aqueous and methanol extracts of *G. farquhariana* were evaluated for antioxidant properties. The results obtained in the study are shown in the table 4.7 and 4.8. The results indicates that the methanol extract shows high antioxidant activity compared to aqueous extracts. Among different sources, seed extract exhibited maximum antioxidant activity compared to leaf and bark extracts. Similar observations pertaining to presence of high antioxidant potentials of seed extracts of *Hippophae rhamnoides* and Sea buckthorn compared to leaf and bark were also made by Sharma et al. (2008) and Michel et al. (2012).

Table 4.7: Antioxidant activity of aqueous extracts of *G. farquhariana*

Mean % inhibition				
Conc. (µg/mL)	**Vitamin C**	**Leaf (Aq)**	**Bark (Aq)**	**Seed (Aq)**
10	39.96±0.35	19.24±0.12	21.68±0.37	25.61±0.47
20	45.25±0.21	23.90±0.22	24.01±0.43	27.16±0.28
40	49.88± 0.72	29.29±0.45	31.79±0.54	33.10±0.52
80	57.19±0.13	32.46±0.35	35.01±0.22	35.60±0.23
100	68.58±0.56	35.74±0.67	39.35±0.34	41.96±0.66
200	75.10±0.34	40.54±0.53	44.36±0.95	45.25±0.76
400	80.27±0.11	43.80±0.83	50.21±0.65	50.88±0.12
800	86.32±0.41	47.52±0.48	52.30±0.25	56.19±0.53
1000	96.34± 0.22	52.80±0.14	56.75±0.16	60.58±0.23

Mean ± SD; N=3

Table 4.8: Antioxidant activity of methanol extracts of *G. farquhariana*

Mean % inhibition				
Conc. (µg/mL)	**Vitamin C**	**Leaf (Met)**	**Bark (Met)**	**Seed (Met)**
10	39.96±0.35	29.35±0.23	31.81±0.54	35.42±0.23
20	45.25±0.21	32.81±0.55	37.30±0.12	40.03±0.22
40	49.88± 0.72	36.01±0.15	40.33±0.21	46.86±0.52
80	57.19±0.13	43.14±0.21	50.76±0.22	54.79±0.42
100	68.58±0.56	51.50±0.52	58.84±0.54	62.83±0.55
200	75.10±0.34	55.35±0.34	66.46±0.72	70.11±0.13
400	80.27±0.11	60.82±0.26	70.38±0.11	74.31±0.21
800	86.32±0.41	64.16±0.18	75.53±0.21	80.95±0.43
1000	96.34± 0.22	71.28±0.55	83.99±0.23	87.99±0.21

Mean ± SD; N=3

The bark and seeds extract in both aqueous and methanol solvents have similar antioxidant activity as that of vitamin C and the seed extracts has demonstrated comparatively high antioxidant activity with compared to leaf and bark extracts. The *G. canarica* seeds extract at the dose of 100µg/mL showed 22.3% free radical scavenging activity of DPPH (Anusha et al., 2019). In the present study at 100µg/mL in methanol seed extract 62.83 % of activity was found. The result was well correlated with amount of phenolics and flavonoid contents present in the plant extracts. The antioxidant activity depends on the phenolic constituents contained in the plant extracts. The reducing power of methanol extracts of *G. farquhariana* seed extract appeared to be higher. Chaithanneya and Bhat (2016) found that phenolic compounds present in the seed methanolic extract (at 500µg/mL showed 31.5%) had higher DPPH scavenging activity compared to aqueous extract. Shazia et al. (2015) reported in *M. fragrans* leaves the highest activity in methanol extract, and the DPPH technique yielded an IC_{50} value of 56.83±5.67 µg/mL.

4.4 MTT assay:

4.4.1 HEK 293 Cell line

A. LEAF

Extract from the leaf in various solvents has shown the following cytotoxicity in the order concerning the percentage of cell viability at 1000 µg/mL. The leaf extract of ethanol is 37.72±4.33µg/mL (IC_{50} 293.52µg/mL; 8.55 µg/mL cisplatin), aqueous is 30.46±4.38 µg/mL (IC_{50} 342.52µg/mL; 2.71 µg/mL cisplatin), methanol is 5.67±0.66 µg/mL (IC_{50} 442.95µg/mL; 1.97 µg/mL cisplatin), acetone is 2.80±0.29 µg/mL (IC_{50} 395.3µg/mL; 4.68 µg/mL cisplatin), chloroform is 2.71±0.03 µg/mL (IC_{50} 552.55µg/mL; 1.65 µg/mL cisplatin). Hence the leaf extract in ethanol has shown the minimum cytotoxicity and the highest cytotoxicity was observed for chloroform extract of the leaf (Figure 4.1).

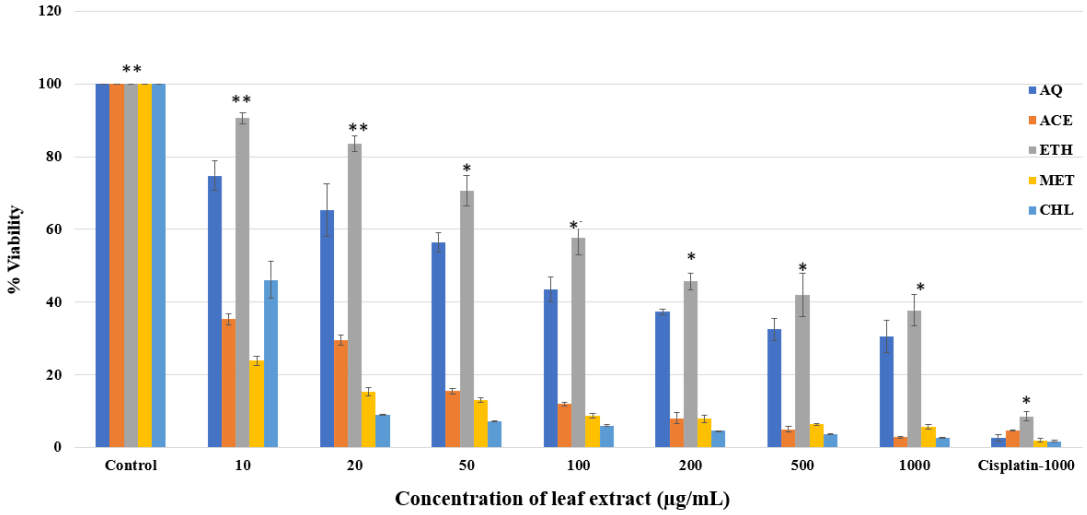


Figure 4.1: Anticancerous activity of the leaf extract of *G. farquhariana* on HEK 293 cell line; Values are in mean ± SD; N=5(p< 0.01; *p< 0.05)**

B. BARK

The bark extract in various solvents had demonstrated cytotoxicity as presented in figure 4.2 in the increasing order of cytotoxicity based on a percentage of cell viability at 1000 µg/mL. aqueous extract of bark 22.77±2.94 µg/mL (IC_{50} 200.09µg/mL; 2.71 µg/mL cisplatin), ethanol extract of bark 17.13±0.63 µg/mL (IC_{50} 310.16µg/mL; 8.55 µg/mL cisplatin), methanol extract of bark 7.72±0.03 µg/mL (IC_{50} 250.15µg/mL; 4.19 µg/mL cisplatin), 7.06±0.80 µg/mL (IC_{50} 213.11 µg/mL; 2.44 µg/mL cisplatin) acetone extract of bark, chloroform extract of bark 2.10±0.67 µg/mL (IC_{50} 390.15µg/mL;

1.65 µg/mL cisplatin). The data proves that the bark extract in the aqueous solvent has shown the minimum cytotoxicity compared to other solvents (Figure 2).

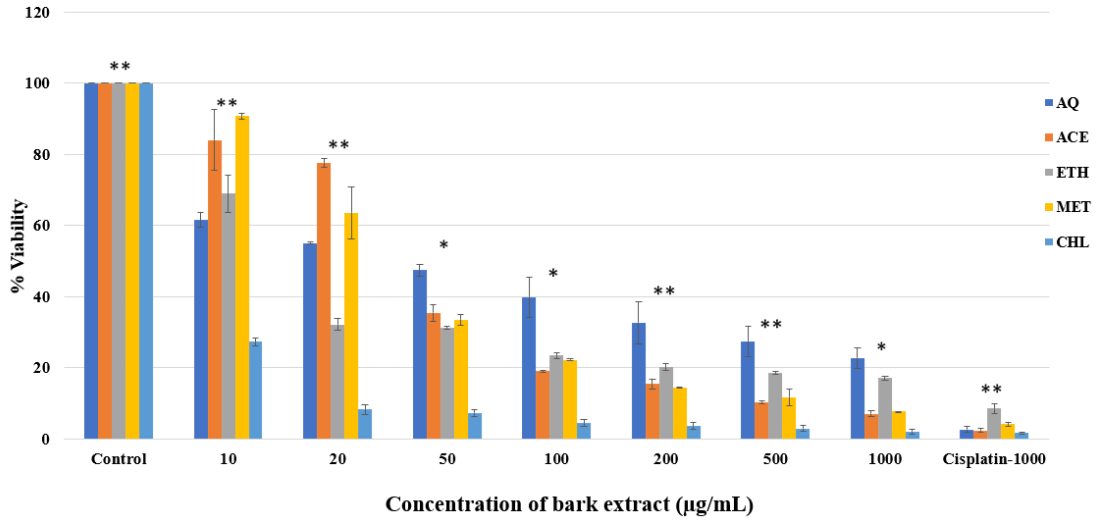


Figure 4.2: Anticancerous activity of the bark extract of *G. farquhariana* on HEK 293 cell line; Values are in mean ± SD; N=5(p< 0.01; *p< 0.05)**

C. SEED

The extract of seed in various solvents has shown considerable variation in the cytotoxicity the data is presented in the order of increasing cytotoxicity based on percentage of viability of the cell at 1000 µg/mL. Aqueous extract of seed 21.611±0.402 µg/mL (IC_{50}197.69µg/mL; 18.051 µg/mL cisplatin), acetone extract of seed 9.106±0.190 µg/mL (IC_{50} 243.59µg/mL; 2.204 µg/mL cisplatin), ethanol extract of seed 6.086±1.561 µg/mL (IC_{50} 310.28µg/mL; 3.013 µg/mL cisplatin), methanol extract of seed 3.489±0.483 µg/mL (IC_{50} 266.69µg/mL; 1.513 µg/mL cisplatin) and chloroform extract of seed 5.466±2.185 µg/mL (IC_{50} 245.75µg/mL; 3.708 µg/mL cisplatin), the data shows that aqueous extract of seed is comparatively less cytotoxic and maximum cytotoxicity of seed is observed in methanol extract (Figure 4.3).

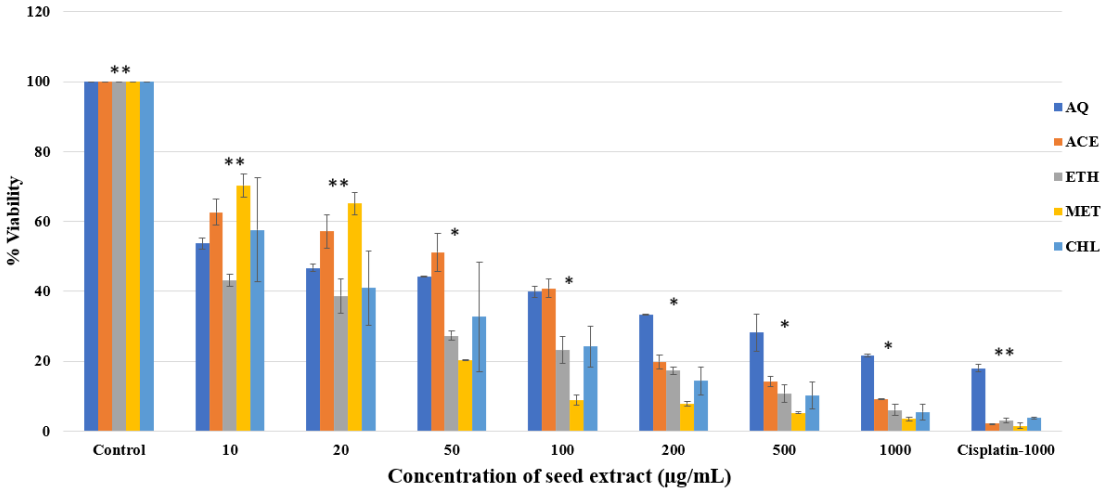


Figure 4.3: Anticancerous activity of the seed extract of *G. farquhariana*on HEK 293 cell line; Values are in mean ± SD; N=5(p< 0.01; *p< 0.05)**

Jafarian et al. (2014) reported that *Cuscuta chinensis* and *Cuscuta epithymum* aerial portions had cytotoxic properties when extracted with chloroform. Many investigations have shown that the constituents' flavonoids, saccharides, alkaloids, lignans, saponins, and resin glycosides are primarily responsible for the pharmacological activity of plants (Biswas et al., 2012; Suresh et al., 2012; Farzami Sepehr et al., 2020). Flavonoids raise the capsase activity and there by significantly decrease cell viability (Farzami Sepehr et al., 2020). The chloroform has lower polarity and it is expected that chloroform extracts less polar compounds. The cytotoxicity of chloroform extracts is expected due to low polar compounds.

To investigate the impact of plant extracts on cancer cell lines, leaf, bark and seed extracts of *G. farquhariana* plants were tested on three malignant cell lines viz., A549, MCF7 and 4T1 cell lines.

4.4.2 A549 Cell line:

A. LEAF

The maximum anticancerous activity on the A549 cell line at 1000µg/mL is observed for the methanol extract of the leaf **(**3.28±0.63 µg/mL). The leaf extract has exhibited different percentages of cell viability for different solvents. The percentage of viability and the anticancerous property at 1000 µg/mL are arranged below according to the anticancerous property in ascending order. Extract in methanol 3.28±0.63 µg/mL (IC_{50} 589.66µg/mL; 1.61 µg/mL cisplatin), 4.65±0.07 µg/mL (IC_{50} 435.47µg/mL;2.16 µg/mL cisplatin) for acetone solvent, 7.46±0.23 µg/mL (IC_{50} 260.29µg/mL; 3.20 µg/mL cisplatin) for ethanol, 8.58±2.05 µg/mL (IC_{50} 630.64µg/mL; 1.106 µg/mL cisplatin) for chloroform, 15.22±1.85 µg/mL (IC_{50}670.99µg/mL; 2.519 µg/mL cisplatin) for extract in aqueous solvent, (Figure 4.4). Madavan et al. (2020) reported that methanolic leaf extract of *Pedalium murex* Linn exhibited maximum anticancer effect on A459 human lung cancer cell line. Alfaifi (2016) observed that methanolic leaf extract of *Kanahia laniflora* showed significant anticancer effect on A459 human lung cancer cell line.

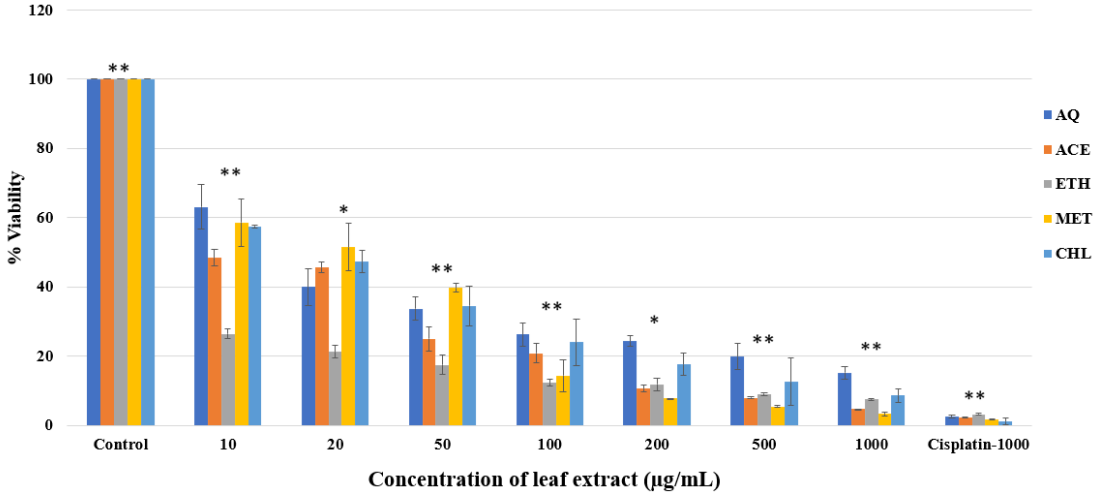


Figure 4.4: Anticancerous activity of the leaf extract of *G. farquhariana* on A549 cell line; Values are in mean ± SD; N=5(p< 0.01; *p< 0.05)**

B. BARK

The chart shows the anticancerous activity of bark extract in various solvents on cell line A549 at 1000 µg/mL. It is found that the extract of bark in chloroform solvent has shown comparatively maximum anticancerous property through a minimum percentage of cell viability of 1.33±0.48 µg/mL. The chart demonstrates that in all solvents the percentage of viability decrease as the concentration increase hence

proving a directly proportional relationship among these. The anticancerous activity in the order of decreased percentage cell viability is as follows at 1000µg/mL.

The extract of bark in chloroform 1.33±0.48 µg/mL (IC_{50} 270.63µg/mL; 1.10 µg/mL cisplatin), 3.28±0.63 µg/mL (IC_{50}295.46µg/mL; 1.61 µg/mL cisplatin) in methanol Extract, 7.81±1.14 µg/mL (IC_{50} 285.96µg/mL; 1.36 µg/mL cisplatin) for bark extract of acetone, 10.73±4.18 µg/mL (IC_{50} 345.55 µg/mL; 3.20 µg/mL cisplatin) in case of extract of bark in ethanol solvent,16.52±5.24 µg/mL (IC_{50} 870.56µg/mL; 2.51 µg/mL cisplatin) for aqueous extract (Figure 4.5). Khanna et al., (2022) reported that chloroform extract of bark of Bauhinia variegate exhibited maximum cytotoxic effect and they attributed that chloroform extracts containing phytochemicals with DNA damaging effects. *Diospyros villosa* chloroform bark extract had showed high potential on A549 cell line also suggested as a potential candidate for a cancer treatment regimen by Adu et al. (2023).

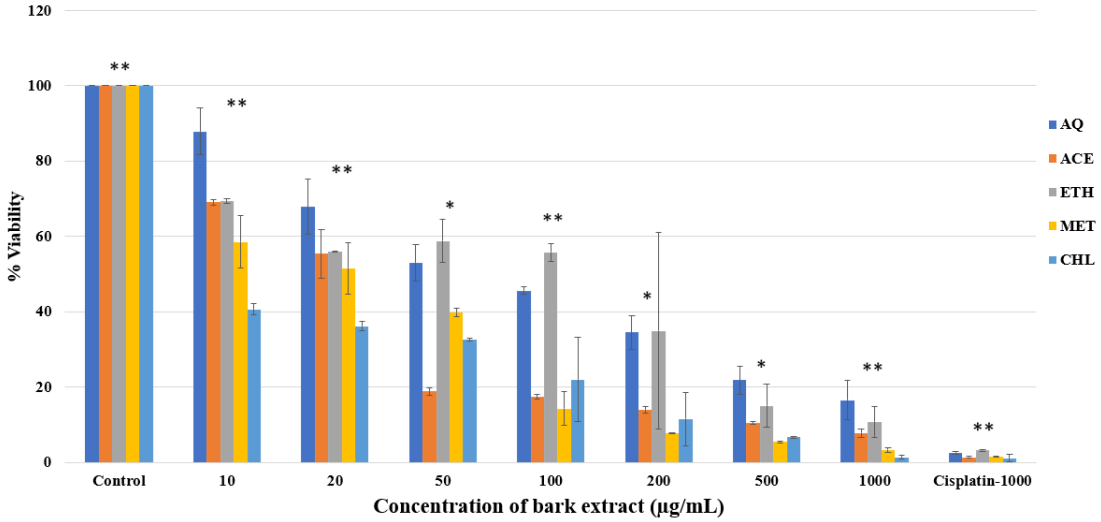


Figure 4.5: Anticancerous activity of the bark extract of *G. farquhariana* on A549 cell line; Values are in mean ± SD; N=5(p< 0.01; *p< 0.05)**

C. SEED

The anticancerous activity of seed extract in various solvents as shown in the chart has shown decreased reported that percentage viability as the concentration of extract increases indicating increased anticancerous properties. It is observed that different solvents have a different pattern of percentage cell viability as the concentration vary. The Highest anticancerous activity exhibited by minimum percentage cell viability is in the ethanol extract of the seed 1.29±0.54 µg/mL (1.58 µg/mL cisplatin). The anticancerous activity among all solvents in 1000 µg/mL is described below for the various solvents used. 1.29±0.54 µg/mL (IC_{50} 235.43µg/mL; 1.58 µg/mL cisplatin) for ethanol solvent, 7.61±02.93 µg/mL (IC_{50} 320.56µg/mL; 2.19 µg/mL cisplatin) for methanol extract, 8.00±0.12 µg/mL (IC_{50} 890.16µg/mL;2.62 µg/mL cisplatin) for seed extract in acetone, chloroform extract of the seed 9.89±0.36 µg/mL (IC_{50} 386.72µg/mL; 1.96 µg/mL cisplatin) 11.57±0.10 µg/mL (IC_{50} 786.26µg/mL; 2.171 µg/mL cisplatin) for aqueous solvent (Figure 4.6).

Kiruthiga, and Sekar (2022) discovered that A549 lung cancer cells reacted most violently to ethanol extracts of *Anisochilus carnosus* bark, and they ascribed this to increased production of reactive oxygen species and changed mitochondrial membrane potential. Ahmed et al. (2020) discovered that *Brassica nigra* (black mustard) seeds significantly inhibited development by decreasing the viability and clonogenic survival of A549 and H1299 cells in a concentration-dependent way. Moreover, it did so

by modulating DNA damage, cell cycle, migration, invasion, proliferation, and apoptosis simultaneously and differently.

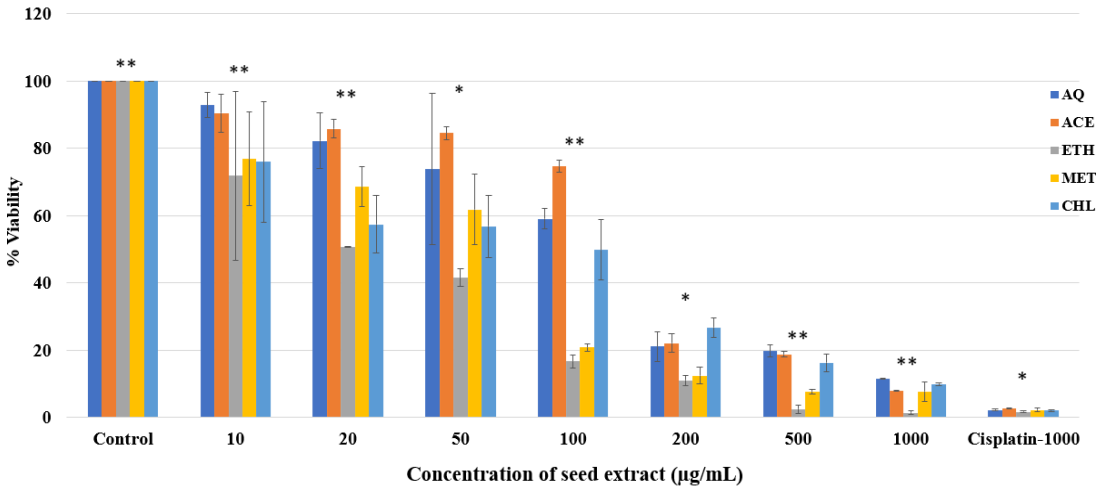


Figure 4.6: Anticancerous activity of the seed extracts of *G. farquhariana* on A549 cell line; Values are in mean ± SD; N=5(p< 0.01; *p< 0.05)**

4.4.3 MCF-7 Cell line:

A. LEAF

Leaf extract of *G. farquhariana* has shown considerable anticancerous activity compared to standard drug. The graph demonstrates that the anticancerous activity grew together with the medication concentration, as indicated by the percentage of viability at the highest concentration. Among the various solvents, it is observed that acetone and methanol extract have shown a progressive increase in anti-cancerous activity. The order of the increase in the activity is as below 2.39±0.51 µg/mL (IC_{50} 198.17µg/mL; 1.36 µg/mL cisplatin) in methanol, 3.46±1.21 µg/mL (IC_{50} 213.45µg/mL; 1.53 µg/mL cisplatin) in acetone, 6.22±1.42 µg/mL (IC_{50} 483.27µg/mL; 1.30 µg/mL cisplatin) in aqueous, 10.31±0.73 µg/mL (IC_{50} 262.74µg/mL; 1.09 µg/mL cisplatin) in chloroform, 19.64±1.09 µg/mL (IC_{50} 332.86µg/mL; 2.25 µg/mL cisplatin) in ethanol (Figure 4.7). The result obtained on effect of leaf extract on MCF-7 cell line is same as A549 cell line. Methanol extract showed maximum cytotoxicity Madavan et al. (2020). Sagadevan et al. (2013) found that as the concentration of drug increases cancer cell inhibition percentage was increased against the MCF-7 cell line in *Andrographis paniculata* and *Cardiospermum halicacabum* methanol leaf extract. *M. concanensis* crude leaf extract exhibits remarkable antitumor activity against MCF-7 cell due to the presence of secondary phytoconstituents in leaf extract by Vijayakumar et al. (2017),

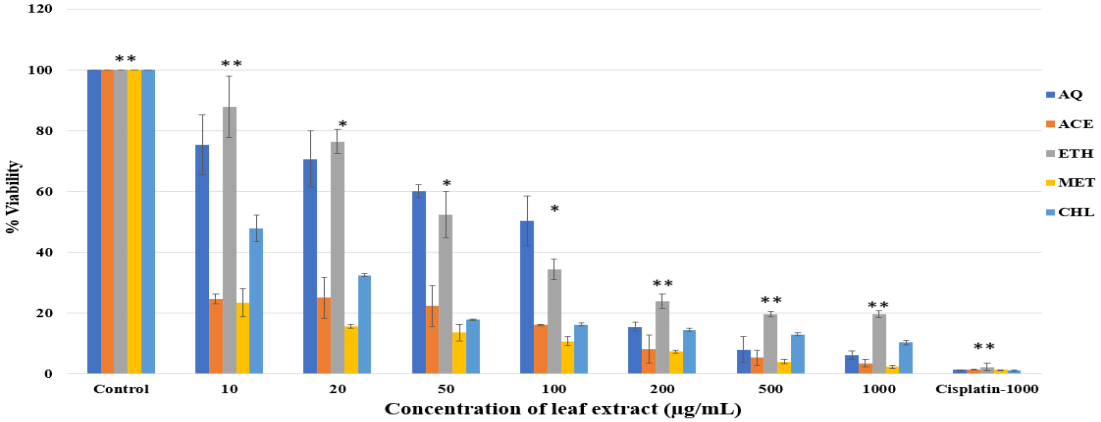


Figure 4.7: Anticancerous activity of the leaf extract of *G. farquhariana* on MCF-7 cell line; Values are in mean ± SD; N=5(p< 0.01; *p< 0.05)**

B. BARK

The figure 4.8 demonstrates that the increased dosage of concentration of the extract in various solvents has increased anticancerous properties, which is directly proportional to the concentration. Among the solvents, aqueous and ethanol solvents have shown comparatively less activity compared to methanol and acetone. It is found that the extract of bark in chloroform has shown the highest activity. The anticancerous property of bark extract at the concentration of 1000 µg/mL in the increasing order is as below 1.50±0.41 µg/mL (IC_{50} 237.27µg/mL; 1.09 µg/mL cisplatin) for chloroform, 5.77±0.73 µg/mL (IC_{50} 260.97µg/mL; 1.70 µg/mL cisplatin) for acetone, 6.92±0.59 µg/mL (IC_{50} 300.27µg/mL; 1.09 µg/mL cisplatin) for methanol, 14.86±0.43 µg/mL (IC_{50} 450.78µg/mL; 1.30 µg/mL cisplatin) for aqueous solvent, 21.77±4.06 µg/mL (IC_{50} 308.94µg/mL; 2.25 µg/mL cisplatin) for ethanol. The result obtained on effect of bark extract on MCF-7 cell line is same as A549 cell line. Chloroform extracts of bark showed maximum cytotoxicity Khanna et al. (2022). The chloroform bark of the birch tree showed The MCF7 cell line's high level of proliferation inhibition was indicated by the cytotoxic activity by Soica et al. (2012).

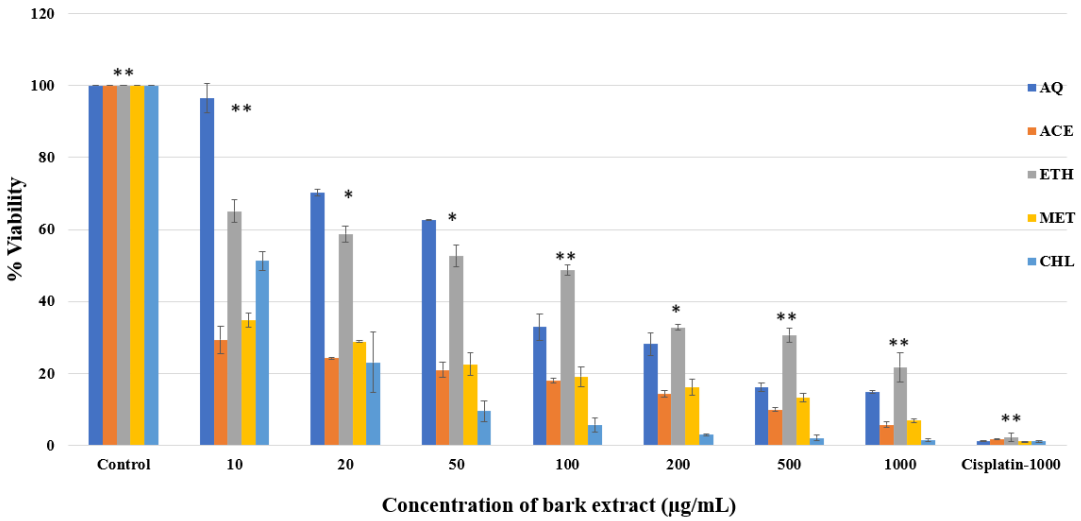


Figure 4.8: Anticancerous activity of the bark extract of *G. farquhariana* on MCF-7 cell line; Values are in mean ± SD; N=5(p< 0.01; *p< 0.05)**

C. SEED

The figure 4.9 depicting the anticancerous activity of the seed extract in various solvents on the MCF7 cell line has shown an increased activity compares the experimental group to the concentration of the drug increased. The concentration of drug is directly proportional to the activity at 1000 µg/mL the various solvents have shown varying effects compared to the standard drug.

The seed extract in chloroform has shown the maximum activity of 1.44±0.20 µg/mL (1.03 µg/mL cisplatin). The anticancerous activity of the extract in various solvents at 1000 µg/mL is shown in the order of increase in the activity 1.44±0.20 µg/mL (IC_{50}175.51µg/mL; 1.03 µg/mL cisplatin) chloroform, 1.86±0.07 µg/mL (IC_{50}190.11µg/mL; 1.03 µg/mL cisplatin) in methanol, 1.90±1.11 µg/mL (IC_{50} 224.24µg/mL; 1.94 µg/mL cisplatin) in acetone, 3.76±0.24 µg/mL (IC_{50} 240.38µg/mL; 1.036 µg/mL cisplatin) in ethanol, 6.82±0.39 µg/mL (IC_{50} 360.93µg/mL; 1.17 µg/mL cisplatin) in aqueous solvent.

As against results obtained in cell line A549, chloroform extract of seed exhibited maximum cytotoxic effect of MCF 7 cell line. The *Nepeta deflersiana* chloroform extract demonstrated that the significant reduction in the MCF 7 and A 549 cells' viability, by altering the cellular morphology in a way depending on concentration and has potent anticancer and pharmacologically active molecules shown remarkable effect *in vitro* by Al-Oqail et al. (2015). Widiyastuti et al. (2018), the avocado seed's ability to cause apoptosis and behave as an anti-proliferative to MCF-7 cell lines was revealed by the avocado seed's chloroform seed extract, *Persea americana* Mill.

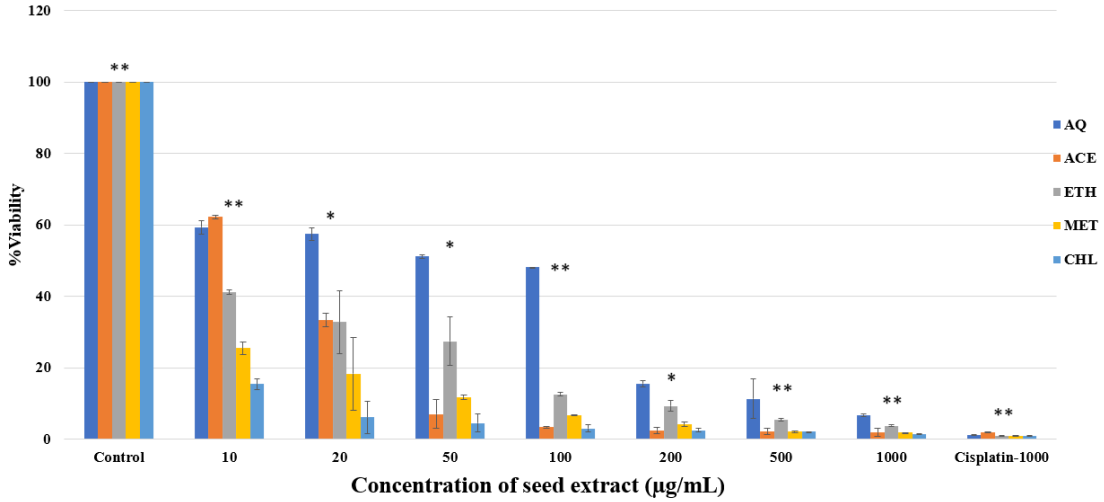


Figure 4.9: Anticancerous activity of the seed extracts of *G. farquhariana* on MCF-7 cell line; Values are in mean ± SD; N=5(p< 0.01; *p< 0.05)**

4.4.4 4T1 Cell line:

The figure 4.10 demonstrates that the methanol and chloroform extracts of the seed when administered on the mice cell line 4T1 have efficiently proved the anticancerous property. The plotted percentage of cell viability against the various concentration of methanol and chloroform extracts shows that the concentration of the medicine and the cell viability % are directly inversely correlated, therefore the anticancerous property.

At 500 µg/mL the percentage of cell viability was 7.44 ±0.41 µg/mL (IC_{50} 195.99µg/mL; 5.4µg/mL cisplatin) for methanol extract and chloroform extract percentage of cell viability was 23.47±8.12 µg/mL (IC_{50} 210.45µg/mL; 5.4µg/mL cisplatin). Kariyil et al. (2021). observed that *Annona muricata* methanolic seed extract showed the effectiveness by caspase-activation and by apoptotic pathway. Mehralikhani et al. (2020), observed that methanolic seed extract showcased noticeably decrease in the expression of dysadherin and Ki67 in 4T1 cell line injected in BALB/c mice model.

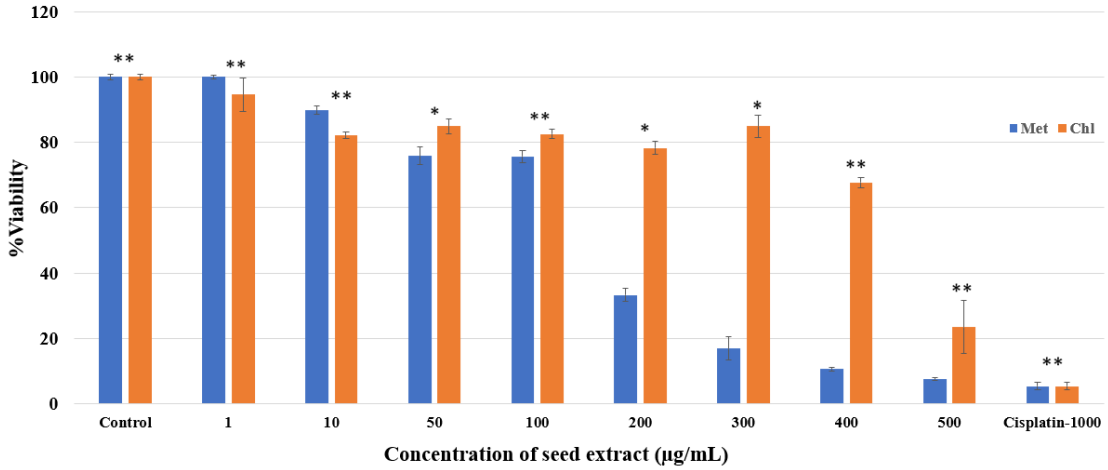


Figure 4.10: Anticancerous activity of the seed extract of *G. farquhariana* on 4T1 cell line; Values are in mean ± SD; N=5(p< 0.01; *p< 0.05)**

The methanol extract of *M. fragrans* caused cell death of Jurkat leukemia T cell line by a mechanism involving SIRTI mRNA down regulation and essential oil of *M. fragrans* on MCF-7and A-357 cancer cell lines to show cytotoxic effect in various concentrations (Chirathaworn et al., 2007; Helen, 2012). The essential oil of nutmeg showed significant inhibition on the expansion of a colon cancer cell line in an *in vitro* study (Piras et al., 2012). The chemopreventive property of mace of *M. fragrans* on DMBA-induced papillomagenesis in the skin of male Swiss albino mice showed a significant reduction in the incidence of skin papilloma in mice as reported by Jannu et al. (1991). The study of chemoprevention action of mace on methylcholanthrene-induced carcinogenesis in the uterine cervix in Swiss albino mice. A dose of 10 mg/mouse/day for 90 days reported a significant decrease in the extent of carcinoma (Hussain and Rao, 1991).

Two phenolic compounds were isolated from fruits of *M. fragrans* which showed significant cytotoxicity activity against the K562 cell line (Duan et al., 2009). When the genotoxic activity of mace of *M. fragrans* on oral cancer cell lines was tested, DNA fragmentation was seen and proved the potential use of mace for anticancerous drugs (Cinthura et al., 2017). The $C_{20}H_{24}O_5$ compound isolated from the Nutmeg plant in n-hexane from root extract when screened for anticancer activity on MCF-7 cell lines found to have moderate anticancer activity (Ginting et al., 2020).

The aqueous and methanolic extracts of *G. farquhariana, M. fatua,* and *Samadera indica* were tested for cytotoxicity on cell line HL- 60, the maximum viability was observed in the methanolic seed extract of *S.indica* at 85.4% and *G. farquhariana* showed considerable percentage viability like 62.9% (Methanolic leaf),73.6% (Aqueous leaf), 65.6% (Methanolic seed),45.9% (Aqueous seed) at 100 µg/mL (Bhat, 2017). In the present study leaf and seed extract of *G. farquhariana* on MCF 7, cell line in aqueous and methanol extracts has shown 10.67 % (Methanolic leaf), 50.42 % (Aqueous leaf), 6.74 % (Methanolic seed), 48.16 % (Aqueous seed) cytotoxicity at 100 µg/mL.

4.5 Animal studies:

The figure 4.11 shows the result of seed extract in methanol and chloroform solvent when administrated on balb/c mice which were subjected to induced tumor growth by administering 4T1 cell line. 4T1cells spontaneously grow from balb/c mammary cancer. These cells are a good model for determining how well anti-cancer medications work (Taheri et al., 2012), which strikingly mimic patients' metastatic

breast cancer and the volume of tumor were recorded on alternative days and compared with cyclophosphamide group with similar treatment p < 9.02E-05 (Taheri et al., 2012). The percentage of volume reduction for methanol group is 94.94 %, chloroform group 53.0% and cyclophosphamide 97.2 % the drug was administered at 25mg/kg.

Hussain and Rao (1991) M. fragrans mace was given orally at a dosage of 10 mg/kg for 7 days using the cervical carcinogenesis model system, and the results showed a reduction of 21.4% compared to the control group's 73.9%. It is extremely significant (P 0.001) that the incidence of cancer has decreased. Thuong et al. (2013) *M. fragrans* DHGA in different concentrations 2mg/kg, 5mg/kg and 10mg/kg was administrated to tumor- bearing mice, at 10mg/kg, the effective reduction of tumor was noticed (74.6 %).

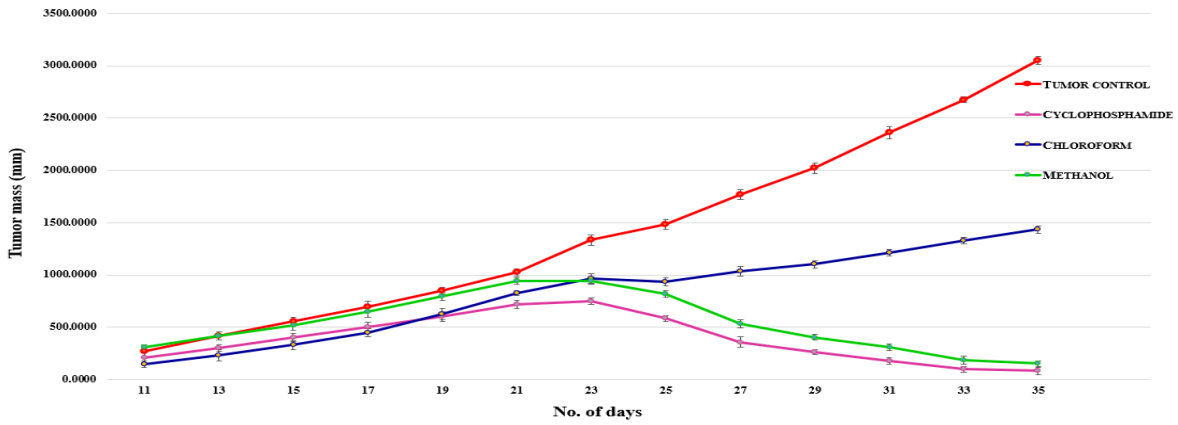


Figure 4.11: Antitumor activity of the seed extract of *G. farquhariana* in tumor control group, methanol group, chloroform group and cyclophosphamide group of 4T1 tumor *in vivo*; Mean ± SE; N=6; (p< 0.01; *p< 0.05)**

The cytotoxic and anticancer effects of *M. fragrans* as a cancer chemo preventive have been explored and indicated that these species might be as good source of cancer chemo preventive drugs, according to prior research by Hussain and Rao (1991). Myristicaceae is an endemic medicinal plant, Traditional uses of Myristicaceae species include the treatment of a wide range of illnesses. These plants also contain a number of biological properties, including antioxidant and cancer chemopreventive properties, recent research have shown that Myristicaceae may have antioxidant, antimutagenic, and cancer chemopreventive properties despite some historical evidence to the contrary regarding its genotoxicity and mutagenicity (Patro et al., 2005; Thuong et al., 2013; Chaithanneya and Bhat, 2016; Bhat 2017; Anusha et al., 2019).

This antitumor activity is attributed to the presence of following antibreast cancer compounds as identified by LCMS in methanol and chloroform crude extract are citalopram, hesperetin, semustine, deoxysappanone B 7,3'- dimethyl ether acetate, hycanthone, buspirone, veratridine, garcinolic acid, dihydrogambogic acid, gambogic acid, amikacin, cholesteryl palmitate, 16:1 cholesteryl ester, vitamin D3 palmitate and stigmastan-3beta-ol, Our results agree with previous research in which the methanol seed extract was found to exhibit a notable growth inhibitory effect on breast tumor in balab/c mice (Amin et al., 2005; Bassiri-Jahromi, 2018; Mehralikhani et al., 2020).

The current findings of increased antioxidant activity in methanol group resulting in significant antitumor activity in methanol group and due to the presence of plant phenolics in *G. farquhariana*

increased antioxidant activity, which has been linked to greater anticancer activity (MCF 7 and 4T1 cell lines) and antitumor activity which might enhanced necrosis and apoptosis pathways (Lande et al., 2015; Marvibaigi et al., 2016). Hence the presence of high phenolic content might responsible for antitumor activity from *G. farquhariana*.

Table 4.9 : ANOVA of antitumor activity of the seed extract of *G. farquhariana* in methanol group, chloroform group and cyclophosphamide group of 4T1 tumor *in vivo*

Groups	Count	Sum	Average	Variance
Methanol	15	6719.525771	447.9683847	76750.19307
Cyclophosphamide	15	5167.190333	344.4793556	58190.82237
Chloroform	15	13648.595	909.9063333	213025.2598

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2719021.574	2	1359510.787	11.72105647	9.02052E-05	3.219942293
Within Groups	4871527.853	42	115988.7584			
Total	7590549.427	44				

The analysis of variance proves that the difference between the groups and within the group is significant since the p value is much lesser than the critical value.

Table 4.10: Histomorphological events in different group of tumors

Events	Control	Methanol	Chloroform	Cyclophosphamide
Tumor necrosis	10%	30%	20%	40%
Myxoid change	Nil	Nil	+	+
Foamy cells	Nil	+	+	+
Lymphoplasmacytic infiltrate	Mild	Mild	Moderate	Mild
Apoptosis	<1/HPF	<1/HPF	1-2/HPF	3-4/HPF

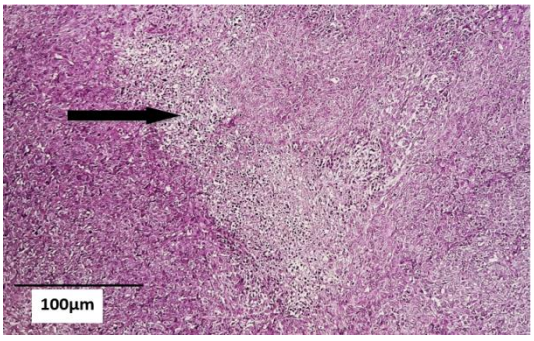


Plate 1: Photomicrograph showing tumor necrosis in the control group (H&E) (HE- 100X)

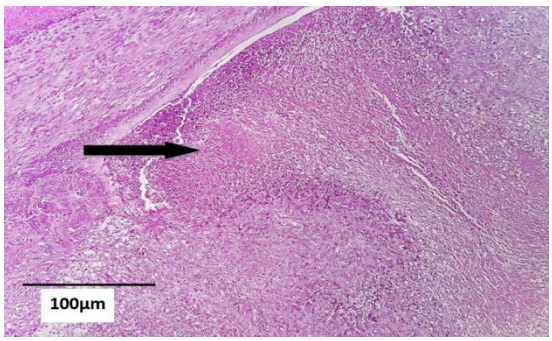


Plate 2 : Photomicrograph showing tumor necrosis (arrow) (arrow) in the cyclophosphamide group (H&E) (HE- 100X)

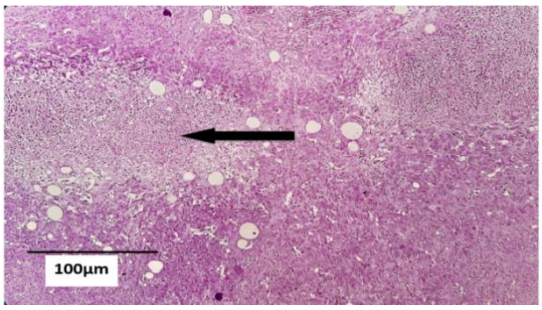


Plate 3: Photomicrograph showing tumor necrosis (arrow) in the chloroform group (H&E) (HE- 100X)

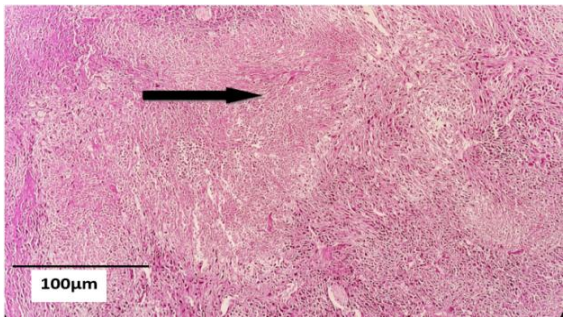
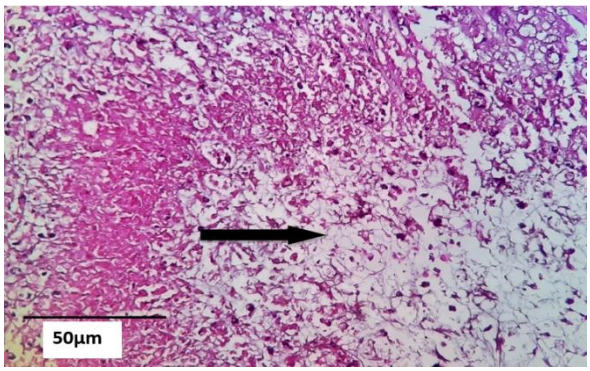


Plate 4: Photomicrograph showing tumor necrosis (arrow)in the methanol group (H&E) (HE- 100X)

Figure 4.12: Tumor necrosis



Pate 5: Photomicrograph showing myxoid change (arrow) in the cyclophosphamide group (H&E) (HE- 400X)

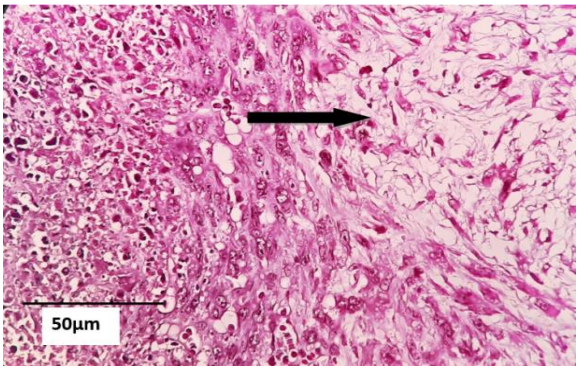


Plate 6: Photomicrograph showing myxoid change (arrow) in the chloroform group (H&E) (HE- 400X)

Figure 4.13: Myxoid change

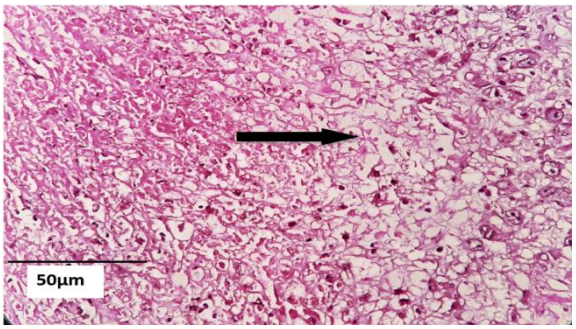


Plate 10: Photomicrograph showing foamy cells (arrow) cyclophosphamide group (H&E) (HE- 400X)

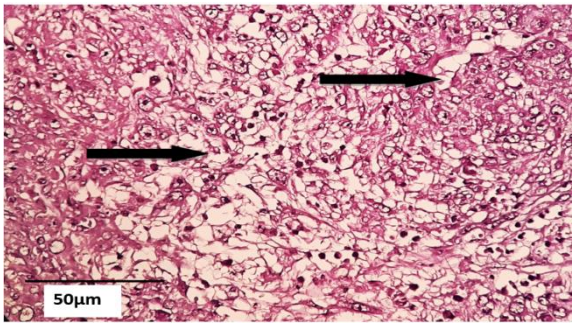


Plate 11: Photomicrograph showing foamy cells (arrow)in the in the chloroform group (H&E) (HE- 400X)

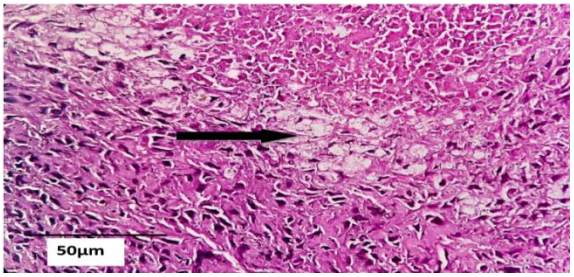


Plate 12: Photomicrograph showing foamy cells (arrow) in the methanol group (H&E) (HE- 400X)

Figure 4.14: Foamy cells

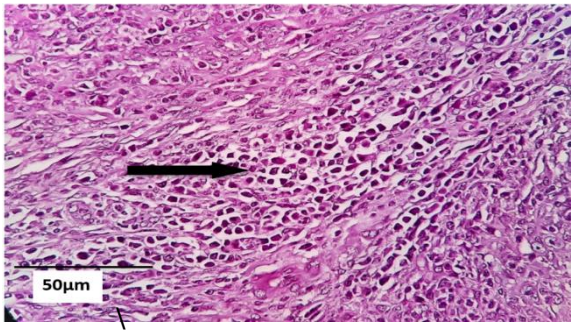


Plate 13: Photomicrograph showing lymphoplasmacytic infiltrate (arrow) in the control group(H&E) (HE- 400X)

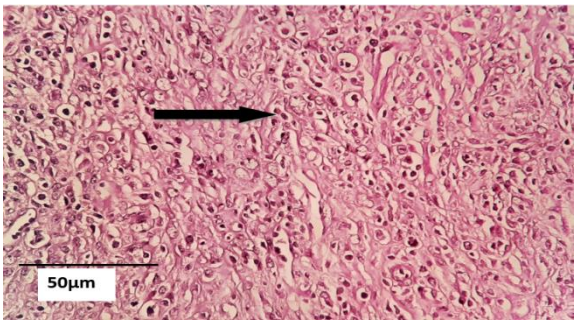


Plate 14: Photomicrograph showing lymphoplasmacytic infiltrate (arrow) in the cyclophosphamide group (H&E) (HE- 400X)

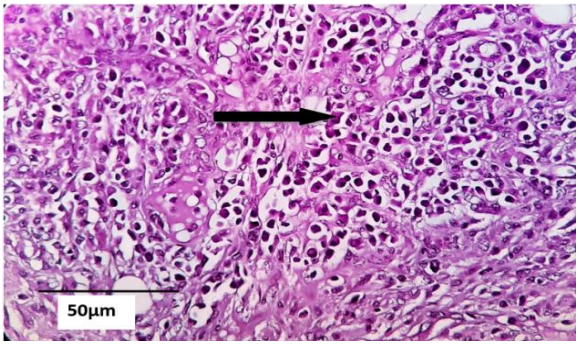


Plate 15: Photomicrograph showing lymphoplasmacytic infiltrate(arrow) in the chloroform group (H&E) (HE-400X)

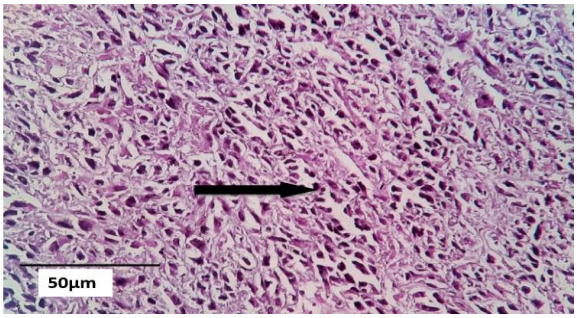


Plate 16: Photomicrograph showing lymphoplasmacytic infiltrate (arrow) in the methanol group (H&E) (HE- 400X)

Figure 4.15: Lymphoplasmacytic infiltrate

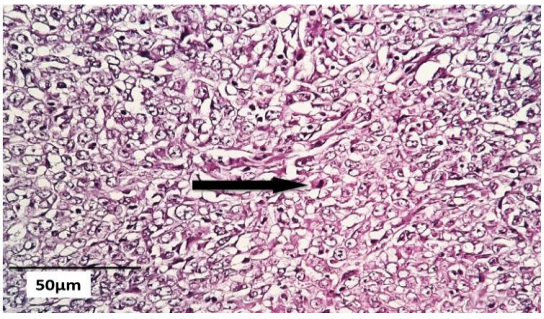


Plate 17: Photomicrograph showing apoptosis (arrow) in the control group (H&E) (HE- 400X)

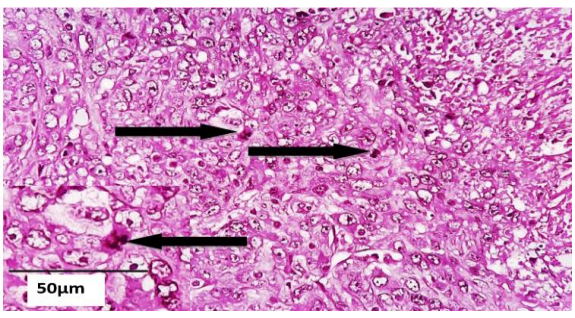


Plate 18: Photomicrograph showing apoptosis (arrow) in the cyclophosphamide group (H&E) (HE-400X)

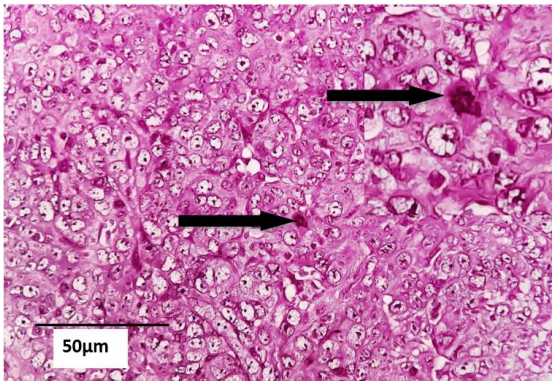


Plate 19: Photomicrograph showing apoptosis (arrow) in the chloroform group (H&E) (HE- 400X)

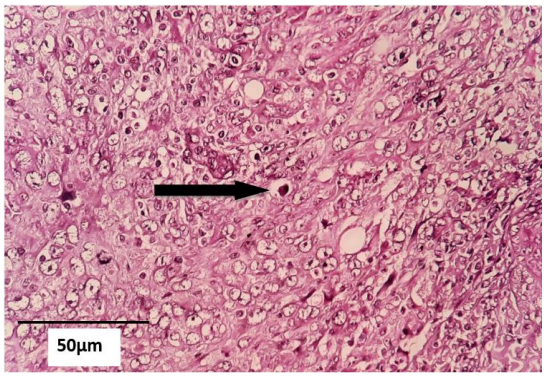


Plate 20: Photomicrograph showing apoptosis (arrow) in the methanol group (H&E) (HE- 400X)

Figure 4.16: Apoptosis

4.6 LC-MS analysis of extracts:

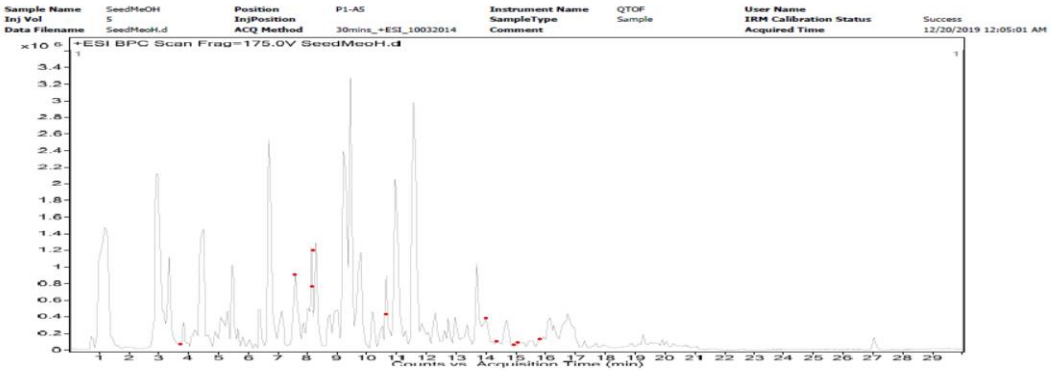


Figure 4.17: LCMS chromatogram of methanol seed extracts of *G. farquhariana* in positive mode

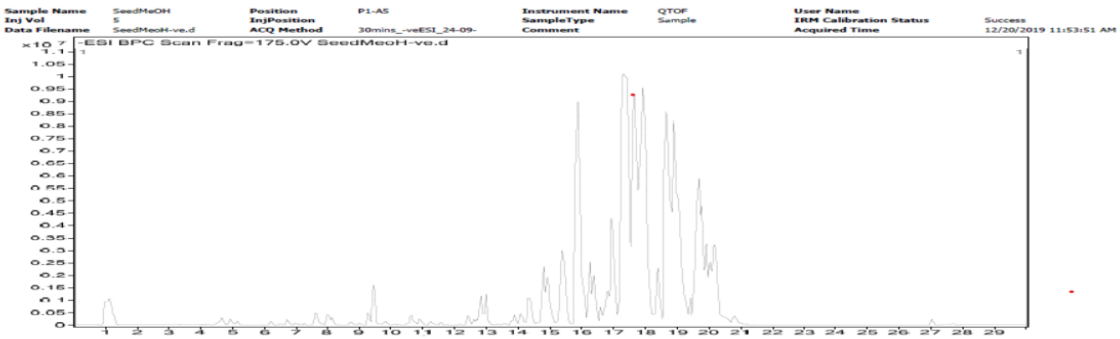


Figure 4.18: LCMS chromatogram of methanol seed extracts of *G. farquhariana* in negative

The present study is the first to investigate and identify compounds of *G. farquhariana* where in seed extract in two solvents, methanol and chloroform was subjected to LCMS. The identified compounds in both positive and negative modes in methanol extract with mass spectrometry attached with liquid chromatography revealed many peaks indicating presence of different bioactive molecules.

The LCMS analysis of methanol seed extract of the plant *G. farquhariana* in both positive and negative mode showed the presence of different compounds table 11. The anticancer and antibreast cancer compounds were identified with their retention time, monoisotopic mass, molecular formula and mass error are presented in The LCMS chromatogram with compound peaks is presented in figure 4.17 and 4.18. Time in minutes is given in X axis and relative abundance in Y axis. In methanol seed extract 18 compounds are identified as anticancer compounds in both positive mode and negative mode among them 11 compounds have proven antibreast cancer property, out of them 10 compounds are extracted in methanol positive mode and 1 compound is extracted in methanol negative mode.

Citalopram reduces hot flashes in breast cancer patience's also acts as antidepressant (Barton et al., 2003; Hickey et al., 2008; Ahmadian et al., 2017; Sakka et al., 2017). Hesperidin interacts with a wide range of known cellular targets and stops the growth of cancer cells by causing apoptosis and cell cycle arrest. Moreover, evidence points to its prospective function in preventing angiogenesis, chemoresistance, and tumour cell metastasis (Choi, 2007; Wolfram et al., 2016; Stanisic et al., 2018; Wang et al., 2021). Semustine are classified as alkylating agents which shows antineoplastic activity (Johnston et al., 1986; Trafalis et al., 2005; Wang et al., 2014; Yao et al., 2022). Deoxysappanone B 7,3'- dimethyl ether acetate anti-angiogenic activity (Bernard et al., 2015; Wang et al., 2019; Hothi et al., 2012; Bailly, 2021).

Hycanthone intercalates into DNA and inhibits RNA synthesis and has shown potential antineoplastic activity (Haq et al., 1980; Chen et al., 2019; Varbanov et al., 2019; Kurniawan et al., 2021). Buspirone has reducing dyspnea in cancer patients during chemotherapy (Cohn et al.,1989; Alexander et al., 2010; Chow et al., 2010; Kumar et al., 2012). Veratridine the plant alkaloid, potentiates cancer chemosensitivity by UBXN2A-dependent inhibition of an oncoprotein (Riganas et al., 2012; Driffort et al., 2014; Dan et al., 2018; Gradek et al., 2019). Garcinolic acid has suppressive antiapoptotic and cytotoxic mechanisms which kills tumor cells (Zhai et al., 2008; Chen et al., 2014; Baruti et al., 2019; Phang et al., 2022). Gambogic acid and Dihydrogambogic acid have potent apoptosis inducing compound also shows impact on tumor size (Wang et al., 2012; Felth et al., 2013; Wang et al., 2015; Kumar et al., 2019; Liu et al., 2020; Yapasert et al., 2020; Majumder et al., 2021).

Arecoline can reduce the expression of miR-22 via repressing p53, which is required to do so in a number of malignancies (Varadharaj et al., 2020; Shih et al., 2021). 4,6-Dioxoheptanoic acid is a potent inhibitor of heme biosynthesis (Tschudy et al., 1983; Ebert et al., 1985). Dihydrokaempferol mechanistically may have anticancer effects primarily via downregulating the expression of proteins related with the development and progression of cancer, coupled with cell cycle arrest, apoptosis activation, and a decrease in the expression

of anti-inflammatory proteins (Ghalib et al., 2013; Zeng et al., 2016;). 4-Hydroxy-3-methoxybenzoic acid acts on apoptosis (Ball et al., 2020; Myint et al., 2021). Swietenine has shown anti-inflammatory action (Moghadamtousi et al., 2013; Nour et al., 2018).

Table 4.11: LCMS analysis of methanol seed extracts of *G. farquhariana* in positive and negative mode

S.No	Name	RT	Monoisotopic mass (M-H-)	Molecular Formula	Mass error (ppm)
1	Citalopram	3.779	324.1638	C20 H21 F N2 O	0.01
2	Hesperetin	7.61	302.0771	C16 H14 O6	6.47
3	Semustine	8.159	247.1034	C10 H18 Cl N3 O2	21.68
4	Deoxysappanone B 7,3'-dimethyl ether acetate	8.263	356.1288	C20 H20 O6	-7.83
5	Hycanthone	10.673	356.1557	C20 H24 N2 O2 S	0.31
6	Buspirone	14.023	385.2515	C21 H31 N5 O2	-9.57
7	Veratridine	14.475	673.3574	C36 H51 N O11	-16.57
8	Garcinolic acid	14.957	646.3155	C38 H46 O9	-2.11
9	Dihydrogambogic acid	15.002	628.3043	C38 H46 O8	3207.04
10	Gambogic acid	15.841	626.2856	C38 H44 O8	3222.24
11	Arecoline	4.466	155.0924	C8 H13 N O2	14.57
12	4,6-dioxoheptanoic acid	5.199	158.057	C7 H10 O4	5.73
13	Dihydrokaempferol	6.956	288.0674	C15 H12 O6	-13.97
14	4-Hydroxy-3-methoxybenzoic acid	7.172	168.0449	C8 H8 O4	-15.78
15	Swietenine	9.074	568.2682	C32 H40 O9	-1.64
16	Digoxigenin bisdigitoxoside	16.353	650.362	C35 H54 O11	7.13
NEGATIVE MODE					
17	Grayanotoxin I	16.717	412.2451	C22 H36 O7	2.54
18	Cinnarizine	17.665	368.2285	C26 H28 N2	-8.93

Digoxigenin bisdigitoxoside induces apoptosis in cancer cells as a result of Nur77 expression and translocation from the nucleus to the cytoplasm (Ren et al., 2018; Patil et al., 2020).

Grayanotoxin I acts as hypoxic tumor cells (Bilir et al., 2018; Abiri et al., 2021). Cinnarizine disturbs the decrease of S-phase and accumulation of cells in G1 phase in the breast cancer cell cycle. It alters the potentials of mitochondrial membranes to release cytochrome C into the cytoplasm and activates caspase-3 to cause cancer cells to undergo apoptosis. According to reports, the activation of apoptosis through the mitochondrial route caused the cell death (Azevedo et al., 2018; Deka et al., 2018; Pontiki et al., 2018; Cristiano et al., 2021).

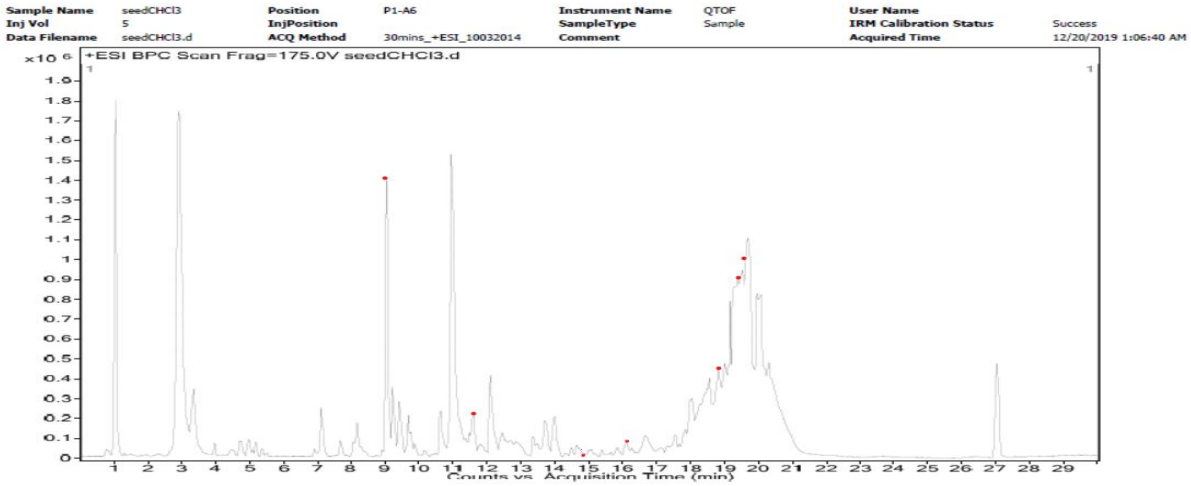


Figure 4.19 : LCMS chromatogram of chloroform seed extracts of *G. farquhariana* in positive mode

The LCMS analysis of chloroform seed extract of the plant *G. farquhariana* in both positive and negative mode showed the presence of different chemical compounds. The anticancer and antibreast cancer compounds were identified with their retention time, monoisotopic mass, molecular formula and mass error (Table 12). The LCMS chromatogram with compound peaks is demonstrated in figure 4.19. Time in minutes is given in X axis and relative abundance in Y axis.

The compounds were identified with mass spectrometry attached with liquid chromatography and revealed many peaks indicating different bioactive molecules. In chloroform seed extract 17 compounds are identified as anticancer compounds in positive mode among them 7 compounds are identified as antibreast cancer compounds, but in chloroform negative mode anticancer compounds were not found.

Table 4. 12: LCMS analysis of chloroform seed extracts of *G. farquhariana* in positive mode

S.No	Name	RT	Monoisotopic mass (M-H-)	Molecular Formula	Mass error (ppm)
1	Amikacin	9.088	585.2866	C22 H43 N5 O13	-1.52
2	Cholesteryl palmitate	11.783	624.5855	C43 H76 O2	-1.47
3	16:1 Cholesteryl ester	19.197	622.5688	C43 H74 O2	0.2
4	Vitamin D3 palmitate	19.604	622.5695	C43 H74 O2	-0.93
5	Stigmastan-3beta-ol	19.8	416.393	C29 H52 O	21.15
6	4-Hydroxy-3-methoxybenzoic acid	7.182	168.0447	C8 H8 O4	-14.76
7	N-Acetyl-beta-D-mannosamine	9.264	221.0897	C8 H15 N O6	1.29
8	Ursinic acid	9.841	276.0981	C15 H16 O5	6.05
9	Ketanserinol	12.106	397.1811	C22 H24 F N3 O3	-2.36
10	Pyrvinium	12.399	381.2209	C26 H27 N3	-1.17
11	Garcinolic acid	14.944	646.3056	C38 H46 O9	13.35
12	Veratridine	16.116	673.3538	C36 H51 N O11	-11.33
13	1,2-dinonadecanoyl-sn- glycerol	18.328	651.5987	C41 H80 O5	1537.62
14	N-Lignoceroylsphingosine	18.681	649.6338	C42 H83 N O3	5.32
15	20:3 Cholesteryl ester	19.497	673.5845	C47 H78 O2	1507.81
16	Flurandrenolide	26.966	436.2306	C24 H33 F O6	-10.27
17	Cholesteryl oleate		650.6069	C45 H78 O2	-10.27

Amikacin has shown migratory inhibition effects on cancer cell (Mahmoud et al., 2020; Mahmoud et al., 2020; Abd El-Halim et al., 2021). Vitamin D3 palmitate Supports the chemotherapy process by active absorption of calcium and phosphorus during the treatment (Bijla et al., 2021; Xia et al., 2022). Stigmastan-3beta-ol is one of the main sterols found in plant cell plasma membranes and is involved in cell proliferation and plasma membrane H+-ATPase activation (Marisa et al., 2021). 4-Hydroxy-3-methoxybenzoic acid reduces cell viability (Ball et al., 2020; Myint et al., 2021).

N-Acetyl-beta-D-mannosamine act as tumor suppressor (Liu et al., 2018; Zheng et al., 2018). Ursinic acid hinders tumour cell transition, restricts cell growth, and triggers apoptosis (Mani et al., 2021; Yang et al., 2021). The principal processes by which pyrvinium works are as follows: I depletion of energy and autophagy; and (ii) inhibition of Akt and Wnt-catenin-dependent pathways. Strong anti-cancer stem cell action has been seen with pyrvinium (Deng

et al., 2013; Wiegering et al., 2014). Garcinolic acid shows suppression of antiapoptotic and cytotoxic mechanisms by which kills tumor cells (Zhai et al., 2008; Chen et al., 2014; Baruti et al., 2019; Phang et al., 2022).

Veratridine is a plant alkaloid, potentiates cancer chemosensitivity by UBXN2A-dependent inhibition of an oncoprotein (Riganas et al., 2012; Driffort et al.,2014; Dan et al., 2018; Gradek et al., 2019). 1,2-dinonadecanoyl-sn- glycerol induces translocation of protein kinase C as well as the inhibition of cell proliferation (Wickramarathna et al., 2015; Czerwińska, et al., 2020). N-Lignoceroylsphingosine responsible for tumor proliferation, progression, and metastasis (Noda et al., 1994; Alrbyawi et al., 2019). 20:3 Cholesteryl ester, cholesteryl palmitate and cholesteryl oleate significantly reduces the proliferation of some solid tumor by reprogramming of lipid/cholesterol pathways in many cancers (Catsoulacos et al., 1993; Ip et al., 2002; Darwish et al., 2018; Jiang et al., 2019; Sarhadi et al., 2019; Vanleeuwen et al., 2020; Ruwizhi et al., 2021; Mahadik et al., 2022). Flurandrenolide shows inflammatory-reduction action (Ai et al., 2018).

5. CONCLUSION

The current study showed that the leaf, bark, and seed aqueous, acetone, ethanol, methanol and chloroform extracts of *G. farquhariana* possess different phytoconstituents with antioxidant and anticancer activity, wherein, methanolic and chloroform seed extract demonstrated strong anticancer activity on MCF 7 cell line and antitumor activity on 4T1 cell line induced tumor on BALB/C mice compared to the other extract in different solvents. Also, by LCMS analysis it's proved the presence of 11 anti-breast cancer compounds in methanol seed extract. This preliminary study has opened a need for further *in vivo* studies to examine various biochemical aspects which might be a step for further conclusions on considering the plant source as a cure for breast cancer.

6. FUTURE STUDIES

The identified anticancer compounds have to be further subjected to screening of molecular level activity on various cancer forms. The 17 antibreast cancerous compounds which are currently used in the treatment may have to be isolated and should be subjected to further pharmacological specific utility along with using various combinations of them in developing alternative drug molecules in the treatment of breast cancer. The extracts in other solvents other than methanol and chloroform used in this study may have to be subjected to analysis of antitumor potency and LCMS analysis. A complete eco profiling of the plant and measures to preserve them with their biodiversity has to be studied and established.

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Paper presentations at International/ National Conferences

1) "Inhibition of migration and apoptosis in breast cancer cells and arrest of tumor progression in an animal model by *Gymnacranthera farquhariana* seed extract" in Alumni sponsored one-day National conference, Alva's College, Moodbidri on 20th February 2020. Won the Best Oral presentation award.

2) *"In vitro* anticancer and cytotoxic activity of extracts of *Gymnacranthera farquhariana* (Hook. f. & Thomson) Warb. - an endemic plant on cancer cell lines" in International e-Conference on Current Science and Technology for Sustainable Society, organized by Aurora's Degree and PG College, Hyderabad and awarded Best Presentation Award on 27th and 28th November 2020

3) Alumni sponsored International e-Conference on "Post-Covid Developments in Biological Sciences" organized by Department of Biotechnology, Alva's College Moodbidri on 5th March 2021 and awarded Best Paper Presentation.

4) "2nd International Pharma e-Conference" organized by Reignite Innovative Conference and Organization Committee Members of Pharma-2021 on April 22-23rd, 2021 at Hyderabad, India and awarded 2nd Best Speaker Award.

5) International e-Conference on "Innovations in Science, Technology, Commerce and Management for the welfare of society" on 29th -31st July 2021, by Aurora's Degree and PG College, Hyderabad and awarded Best Presentation Award.

6) Participated and presented a paper in One day International seminar on "Recent Innovations in Science and Technology for the Sustainable Ecosystem" organised by Department of Botany and Environmental Studies, AVK College for Women, Hassan on 2nd July 2022 and secured First prize in the oral presentation.

7) Participated and presented a paper in National Seminar on "Research Methodology" Alva's Samveda – 2022 jointly organized by Alva's Traditional Medicine Archive (ATMA) and Research Centre and Himalaya Wellness Company, Bangalore on 30th July 2022 and secured First prize in an oral presentation.

Research publications arising out of the thesis

1) Gagana, B., and Rama Bhat, P. (2020). *In vitro* anticancer and cytotoxic activity of extracts of *Gymnacranthera farquhariana* (Hook. f. & Thomson) Warb. - an endemic plant on cancer cell lines. *Journal of Advanced Scientific Research*, 11,17-22.

2) Gagana, B., and Rama Bhat, P. (2022). Anticancer and cytotoxic activities of *Gymnacranthera farquhariana* (Hook. f. & Thomson) Warb. extracts on cancer cell lines. *Dickensian Journal*,22 (5) 1961-1971.

3) Gagana, B., and Rama Bhat, P. (2022). Phytochemical analysis and anticancer activity of leaf, bark, and seed extracts of *Gymnacranthera farquhariana* (Hook. f. & Thomson) Warb. *Medicinal Plants*,14 (4), 513-523. (Accepted, in press).

Research guide **Research Schola**